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OPTIMIZATION OF INTERMITTENT PREVENTIVE THERAPY FOR MALARIA DURING PREGNANCY: EFFECTIVENESS OF DIHYDROARTEMISININ-PIPERAQUINE VERSUS SULFADOXINE-PYRIMETHAMINE

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The cover picture illustrate mosquito transmitting malaria to pregnant woman and red blood cells infected with malaria parasites

Optimization of Intermittent Preventive Therapy for Malaria during Pregnancy: Effectiveness of Dihydroartemisinin-piperaquine versus Sulfadoxine-pyrimethamine

THESIS FOR DOCTORAL DEGREE (Ph.D.)

The public defense will be held at Yxlan room Novum 6A, Karolinska University Hospital Huddinge, on Friday June 18th 2021, 9:30am.
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To my family and friends

‘‘Kind words can be short and easy to speak, but their echoes are truly endless. Every time you smile at someone, it is an action of love, a gift to that person, a beautiful thing’’ Mother Teresa-Calcutta.

ABSTRACT

Malaria is a Tropical disease caused by different parasites species of genius *Plasmodium*. Because of pregnancy associated lowered immunity, women who are pregnant are at higher risk to malaria infection than non-pregnant women. Malaria infection in pregnancy is public health problem particularly in sub-Saharan Africa causing anemia to mothers, premature-birth, stillbirth and low birth weights (LBW) . To minimize the risk of malaria and its associated poor outcomes, the World Health Organization (WHO) endorsed preventive policy for all pregnant women residing in endemic areas. The policy include receiving intermittent preventive treatment in pregnancy (IPTp) with a drug sulfadoxine-pyrimethemine (SP) each month commencing from early second trimester, owning and using insecticide treated bed nets (ITNs) and timely symptomatic malaria case treatment. The rapidly growing resistance of malaria-causing organisms (*P. falciparum)* to SP rises questions on the potency of IPTp with SP (IPTp-SP). This activated the exploration of an alternative drug for IPTp. In endemic areas with high malaria transmission intensity, IPTp with dihydroartemisin-piperaquine (IPTp-DHP) given early during the second trimester (≤20 weeks) was reported to be superior to the standard IPTp-SP for protection against parasitemia and placental malaria but not negative birth outcomes. However, variability in malaria transmission intensity is a crucial factor which could possibly impact the outcomes of an intervention. In addition, the association between piperaquine pharmacogenetics and pharmacokinetics with IPTp-DHP outcomes were lacking in the literature.

This thesis investigated the effectiveness of the standard monthly IPTp-SP versus IPTp-DHP for protection of malaria and negative birth outcomes from a setting with moderate malaria transmission intensity. We recruited women on their really timing to the first ANC, thus pregnant women both on their second and third trimesters were included.

Firstly, we explored the burden of asymptomatic parasitemia, anemia and associated factors among pregnant women attending their first antenatal care (ANC) (**Paper I**). We found that, 36.4% of women had asymptomatic parasitemia associated with anemia at their initial ANC. Women who are pregnant for the first time and adolescent were found to have higher risk of asymptomatic parasitemia and anemia compared to women who are pregnant for more than once and adult, respectively.

In **paper II**, we prospectively evaluated the safety and effectiveness of the standard IPTp-SP for preventing malaria in pregnant women and negative birth outcomes . We observed that, one sixth (16%), one fifth (20.9%), and one fourth (26.5%) of women receiving the standard monthly IPTp-SP had parasitemia during pregnancy, any parasitemia at delivery and any adverse birth outcomes, respectively. We also found 9.4% of women had histopathological placental malaria associated with negative birth outcomes. In addition, significant association between three and above doses of monthly IPTp-SP with improved birth weight was found as compared to less than three IPTp-SP doses.

In **paper III**, the effectiveness of the standard monthly IPTp-SP for prevention of malaria and associated negative birth outcomes was compared with monthly IPTp-DHP in a randomized controlled trial. From a moderate *P. falciparum* transmission area, IPTp-DHP was found to be superior to IPTp-SP for prevention of symptomatic malaria and parasitemia during pregnancy, parasitemia and placental malaria at delivery. In addition, this thesis observed the superior impact of IPTp-DHP on birth weight as compared to the standard IPTp-SP for the first time.

Finally, we assessed piperaquine pharmacogenetics and day-7 pharmacokinetics with their relevance on IPTp-DHP outcomes (**Paper IV**). We found Day-7 piperaquine concentration increasing significantly after each monthly IPTp-DHP. Women with day-7 piperaquine concentrations <30ng/mL were found to have significantly higher risk of having parasitemia during pregnancy as compared to those with higher concentration (≥30ng/mL). Also, carriers of defective *CYP2C8* allele were found to have significantly lower day 7 piperaquine concentration overtime as compared to wild type.

In **conclusion,** Asymptomatic malaria and associated anemia at first ANC visit is common in sub-Sahara Africa particularly among primigravida and adolescent pregnant women. In addition, we reported considerable burden of parasitemia, placental malaria and associated negative birth outcomes among women receiving the standard IPTp-SP. This thesis also reaffirmed the role higher doses of IPTp-SP in improving birth weight from areas with high *P. falciparum* resistance to SP. Notably, we reported for the first time the superior effect of IPTp-DHP for prevention of malaria in pregnancy and improving birth weight as compared to IPTp-SP from a setting with moderate malaria transmission intensity. We also reported significant association between lower day-7 piperaquine concentrations with the risk of malaria during pregnancy. Lastly, we found significant association between *CYP2C8* genotypes with day-7 piperaquine pharmacokinetics.

LIST OF SCIENTIFIC PAPERS

I. **Mlugu EM**, Minzi O, Kamuhabwa AAR, Aklillu E. Prevalence and Correlates of Asymptomatic Malaria and Anemia on First Antenatal Care Visit among Pregnant Women in Southeast, Tanzania. Int J Environ Res Public Health. 2020; 17(9):3123. doi: 10.3390/ijerph17093123.

II. **Mlugu EM,** Minzi O, Asghar M, Färnert A, Kamuhabwa AAR, Aklillu E. Effectiveness of Sulfadoxine-Pyrimethamine for Intermittent Preventive Treatment of Malaria and Adverse Birth Outcomes in Pregnant Women. Pathogens. 2020; 9(3):207. doi: 10.3390/pathogens9030207.

III. **Mlugu EM,** Minzi O, Kamuhabwa AAR, Aklillu E. Effectiveness of intermittent preventive treatment with dihydroartemisinin-piperaqunine against malaria in pregnancy in Tanzania: A Randomized Controlled Trial. Clin Pharmacol Ther. 2021. doi: 10.1002/cpt.2273.

IV. **Mlugu EM**, Minzi O, Johansson M, Kamuhabwa AAR, Aklillu E. Pharmacogenetics and Pharmacokinetics of Piperaquine and its Association with Intermittent Malaria Preventive Therapy outcome in Pregnancy: A prospective cohort study.
(Manuscript)

CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin-based Combination Therapy
ADR	Adverse Drug Reaction
AIDS	Acquired Immunodeficiency Syndrome
AL	Artemether-Lumefantrine
ANC	Antenatal Care
ANOVA	Analysis of Variance
ARVs	Antiretroviral Drugs
AUC	Area Under the concentration Curve
CDC	Center for Disease Control
CRF	Case Report Form
CYP	Cytochrome P450
DHP	Dihydroartemisinin-Piperaquine
DNA	Deoxyribo-Nucleic Acid
DOT	Direct Observed Therapy
EDTA	Ethylenediaminetetraacetic Acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRP2	Histidine Rich Protein 2
IPTp	Intermittent Preventive Treatment in pregnancy
ISTp	Intermittent Screening and Treatment in Pregenancy
ITN	Insecticide Treated bed Net
KI	Karolinska Institutet
LBW	Low Birth Weight
LC	Liquid Chromatography
MDGs	Millennium Development Goals
MHCDGEC	Ministry of Health, Community Development, Gender, Elderly and Children
mRDT	Malaria Rapid Diagnostic Test
MS	Mass Spectrometry
MUHAS	Muhimbili University of Health and Allied Sciences
NIMR	National Institute for Medical Research
NMCP	National Malaria Control Programme
PCR	Polymerase Chain Reaction
PE	Protective Efficacy

PG	Pharmacogenetics
PK	Pharmacokinetics
SDGs	Sustainable Development Goals
SP	Sulfadoxine-Pyrimethamine
SPSS	Statistical Package for Social Sciences
SSA	Sub-Saharan Africa
THMIS	Tanzania HIV/AIDS and Malaria Indicator Survey
UPLC-MSMS	Ultra high Performance Liquid Chromatography tandem-Mass Spectrometry
WHO	World Health Organization

1 INTRODUCTION

1.1 AN OVERVIEW OF MALARIA

Malaria is a parasitic disease mostly occurring in the tropical and sub-tropical regions and caused by the microorganisms belonging to genus Plasmodium. Species of Plasmodium known to be important causes of malaria disease in human are currently six [1]. However, *Plasmodium falciparum* is the most common parasite associated with disease morbidity and mortalities in sub-Sahara Africa. *P. malariae* and the two species of *P. ovale (P. ovale curtisi, P. ovale wallikeri)* are less common causes of disease and its consequences. On the other hand, *P. vivax* is the most common specie causing malaria in Latin America and to some extent in some parts of Africa and Southeast Asia. In recent years, a zoonotic parasite of simian, known as *Plasmodium knowlesi* appeared to be a key cause of human malaria especially in Southeast Asia [2,3].

Malaria parasites are transmitted to humans by female mosquitoes of the genus Anopheles. In Africa, a number of anopheles species are responsible for the transmission of malaria, but three species are the most common, namely; *An. gambiae*, *An. arabiensis* and *An. funestus* [4]. Among these three primary dominant vector species, *An. gambiae* and *An. funestus* tend to bite indoor and during the night when people are asleep [4]. On the other hand, *An. arabiensis* easily adopt to drier environments and tend to feed outdoors [4].

1.1.1 *Plasmodium falciparum* life cycle

Plasmodium parasites life cycle alternate between female anopheles mosquitoes and human host (**Figure 1**). Usually, parasites in the stage known as sporozoites are released and transmitted to human through the dermis by the mosquito during feeding. From the dermis, sporozoites gain access to the blood through cellular traversing [5] and migrates to the liver cells within some minutes to few hours [6]. The invasion of sporozoites to hepatocytes is facilitated through binding of parasites to hepatocytes surface proteins known as tetraspanin CD81 and scavenger receptor B1 (SR-B1) [7]. After 2-10 days since entry, parasites mature in the hepatocytes and create parasite filled vesicles well known as merosomes. Merosomes protect the parasites against human host immunity and ensures their migration into the red blood cells. Merosomes are then released into the liver blood vessels known as sinusoids [8]. Then, merosomes rupture and release malaria parasites called merozoites in this stage. Merozoites rapidly invade erythrocytes through binding to receptors in human red blood cells using parasites' erythrocyte binding ligands (EBLs) [9]. Within erythrocytes, each individual merozoite divides asexually to form a colony of parasites known as schizont. After 48 hours since erythrocytes infection, the schizont ruptures, release several merozoites (16 to 32) and each merozoite initiate a new infection cycle (**Figure 1**). Furthermore, the rupture of schizont is associated with the clinical symptoms of malaria. Some of the parasites in infected red blood cells commit to sexual development where they form male and female gametocytes. The maturity of *P. falciparum* gametocytes occurs in the bone marrow and takes about 11 days since commitment has initiated [10]. This stage determines

the transmission of parasites to mosquito and is one of the key intervention point through transmission blocking medicines or vaccines. When mosquitoes feed on human blood, mature gametocytes are taken to the mosquito. Finally, male and female gametocytes fuse in mosquito and the cycle begins again (**Figure 1**).

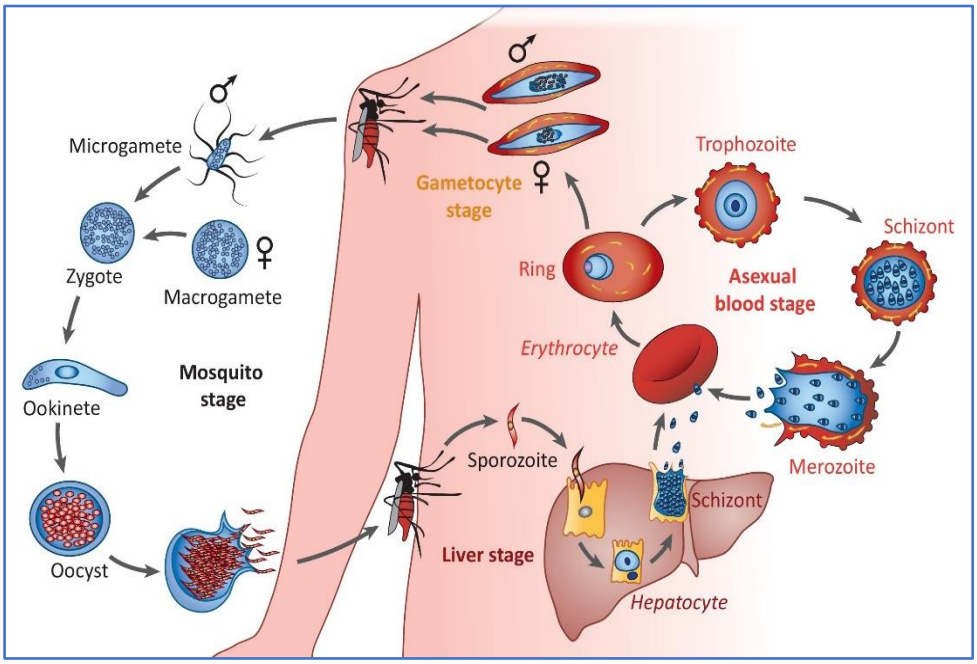

Figure 1. Representation of *Plasmodium falciparum* life cycle both in human and in mosquito hosts. Adapted with permission form Maier AG. et al. (2018) [11].

1.1.2 Immunity against Malaria

Human immunity against malaria involves both innate and adaptive mechanisms. Innate immune reaction responding to malaria infection is a first-line impeding the development of malaria parasite and activate defensive immunity via adaptive mechanism. During malaria infection, parasite-infected erythrocytes, malaria pigments, merozoites, and complexes associated immune are phagocytized by dendritic cells, macrophages and neutrophils [12]. This occurs both in hepatocytes and in erythrocytes and modulates immune responses to malaria [13]. However, during blood stage infection, when macrophages engulf infected red blood cells, malaria pigments and merozoites, they cannot secrete mediators such as chemokines and cytokines; thus, they become immunosuppressive and dysfunctional [14,15]. Therefore, in this stage dendritic cells have the role to release these mediators reacting to malaria parasites, as well as interacting with the adaptive and innate immune mechanisms.

During pre-erythrocytic stage, cell-mediated immunity with CD8 T cells and neutralizing antibodies are involved. The parasites' surface proteins known as circumsporozoite proteins (CSPs) are important target antigens. The primary role of antibodies is to counteract sporozoites and inhibit them from infecting liver cells, whereas CD8 T cells initiate cytotoxic effects and destroy the parasites [16]. In fact, CSPs are key targets for malaria vaccine development. For effective protection against malaria infection, high amount of

antibodies against CSPs are required. Unfortunately, a normal infection induces insufficient CSP-specific antibodies [17]. In addition, the protection against parasite infection is further compromised when the blood cycle begins. In this case, cell-mediated responses in the liver is suppressed due to infection in erythrocytes [18].

During infection stage in erythrocytes, there is no cytotoxic response because red blood cells lack antigen-presenting mechanism [19]. Thus, protection via antibody-mediated mechanism is a critical constituent of naturally acquired immunity in this stage. The family of *P. falciparum* erythrocyte membrane proteins (*Pf*EMPs) are the main earmarks of antibodies against malaria infection in blood-stage infection [20]. The likely antibody effector mechanisms include; increased clearance of infected erythrocytes through antibodies-antigens reaction, obstructing infection of new red blood cells, or antibody-mediated cellular killing [21].

After an acute malaria infection antibody titer rapidly decline to low levels. However, adult people living in endemic areas usually maintain high antibody levels due to repeated episodes of infections [22]. This explains why people living in low-endemic area have poor immunity against malaria compared to those living in moderate and high-endemic areas. However, this immunity is not a sterilizing type but rather keeps the parasites at low density unable to elicit symptoms. At any time when immunity is compromised the parasites balanced at low density can multiply and cause symptomatic infection a phenomenon called recrudescence. Usually, a term premunition is used to describe the balance between malaria infection and human immunity. Premunition is hypothesized to occur as a result of interaction between protective antibodies and monocytes leading to production of soluble mediators which block the division of intra-erythrocytic parasites at the trophozoite stage [21]. This might explain partly why asymptomatic malaria cases are common in endemic areas. In addition the immunity acquired with repeated malaria exposure may not protect individuals from being re-infected.

1.2 THE GLOBAL BURDEN OF MALARIA

Malaria is still a public health problem causing ill health and associated mortalities, particularly in resource-limited countries, despite the substantial decline in burden globally. The beginning of a new millennium in the year 2000 is particularly important, giving the new direction in the fight against malaria globally. The Millennium Development Goals (MDGs) brought forth during this year stimulated political support and great financial commitments, which led to the rise of new innovative strategies for the control of malaria [23]. Indeed, this resulted in substantial achievements in the war against malaria. For instance, worldwide, malaria annual incidence and death rate have respectively reduced by 36% and 60% from the year 2000 to 2016 [24].

In 2015, the ‘‘Global Development Sustainable Goals’’ (SDGs) came forth to sutain the achievements of MDGs. During this year, the World Health Organization (WHO) endorsed the special strategy for malaria control known as ‘‘the Global Technical Strategy for Malaria 2016–2030 (GTS 2016-2030)’’ [25]. In fact, the GTS 2016-2030 was the special

interpretation of the malaria-specific global ‘‘SDG 3.3’’ that aimed to end in addition to malaria, the epidemics of acquired immunodeficiency syndrome (AIDS), tuberculosis, and neglected tropical diseases (NTDs) by 2030 [26]. The GTS 2016-2030 was introduced to sustain the malaria control achievements, aiming to lower further the incidence of malaria and malaria-associated death rates by 90% at the end of 2030 compared to the levels of 2015 [25]. By the end of 2020, the GTS aimed to reduce malaria incidence and mortality rates by at least 40% compared with 2015 levels [25]. However, the global annual estimated malaria cases increased from 218 million cases in 2015 [27] to 231 million cases in 2017 [28], 228 million cases in 2018 [29] and 229 million cases in 2019 [30]. On the other hand, malaria related mortality decreased by 10% from 453,000 deaths in 2015 [27] to 405,000 deaths in 2018 [29] and 409,000 in 2019 [30]. The increase in global malaria burden in the past three years suggest that the 2020 targets for GTS 2016-2030 are less likely to be attained. The poor progress in malaria reduction might be due to sub-optimal control strategies, financial constrains or other social determinants which really need to be addressed.

Above 90% of global malaria cases and malaria-associated mortality occur in sub-Saharan Africa affecting mostly pregnant women and children (**Figure 2**). In sub-Saharan Africa, estimates indicate that, more than 33 million pregnant women are at risk for malaria infection [31]. About 11 million and 12 million pregnant women had malaria infection in 2018 and 2019, respectively [29,30]. Following the stall in the progress for malaria reduction, WHO in collaboration with Roll Back Malaria (RBM) program launched a country-specific approach in 2018 to help countries with a high burden of malaria to get back to the track towards GTS targets [32].

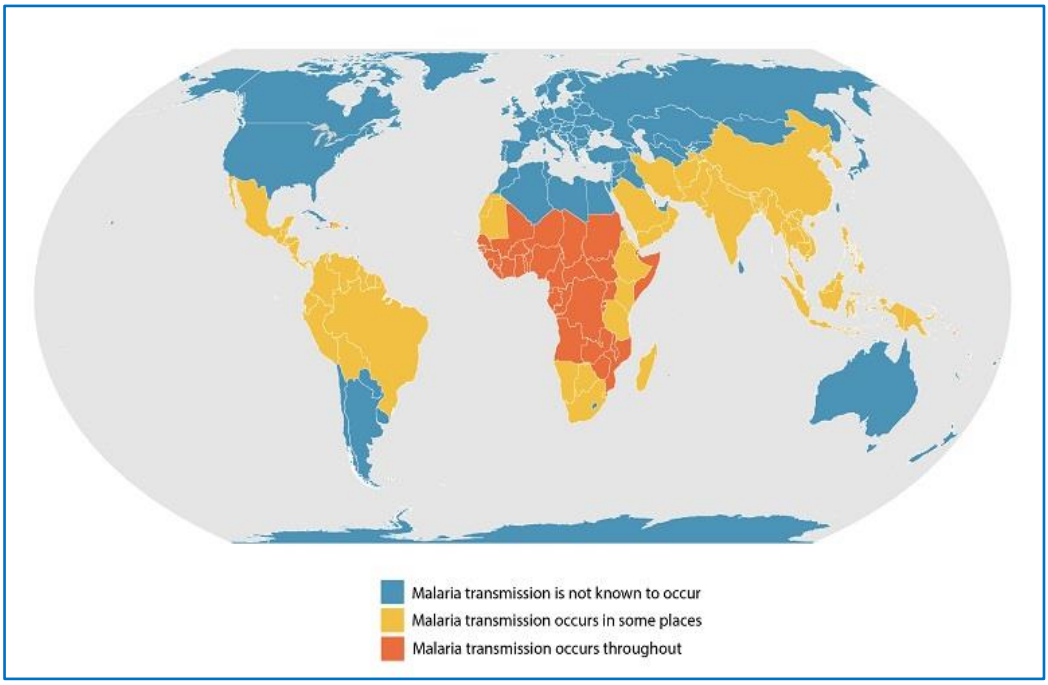

Figure 2. Global estimated transmission of malaria as of 2020. Adapted from CDC (2020) [33].

1.3 MALARIA BURDEN IN TANZANIA

In Tanzania, the transmission of malaria is diverse. Some areas have high transmission intensity while others have moderate, low malaria and very low transmission intensities (**Figure 3**). Zones of high transmission intensity are found on the northwest and southern part of the country. The zone of moderate transmission is located on the coastal area, whereas low and very low transmission intensity are found on the middle and north eastern part of the country. Tanzania is currently listed as one of the 11 high malaria burden countries and contributed more than 6 million cases of the global malaria burden in 2018 and 2019 [29,30]. Nevertheless, the malaria burden in Tanzania decreased substantially within three years from 2014 (15%) [34] to 2017 (7.3%) [35]. The substantial reduction of malaria burden has been contributed by the optimization of malaria control strategies. This finding could suggest that Tanzania is making good progress towards malaria elimination. However, the WHO estimated an increase in malaria cases from Tanzania in 2018 and the same estimate was reported in 2019 [29,30], indicating a turning back. Equally, the proportion of pregnant women testing positive during ANC visit only decreased by 1.4% between 2014 to 2018 [36]. In 2010, a mathematical model estimated that, about 500,000 pregnant women in Tanzania are exposed to malaria infection [37]. Persistent malaria among pregnant women despite the decline in the general population may indicate the requirement for additional control approaches among pregnant women.

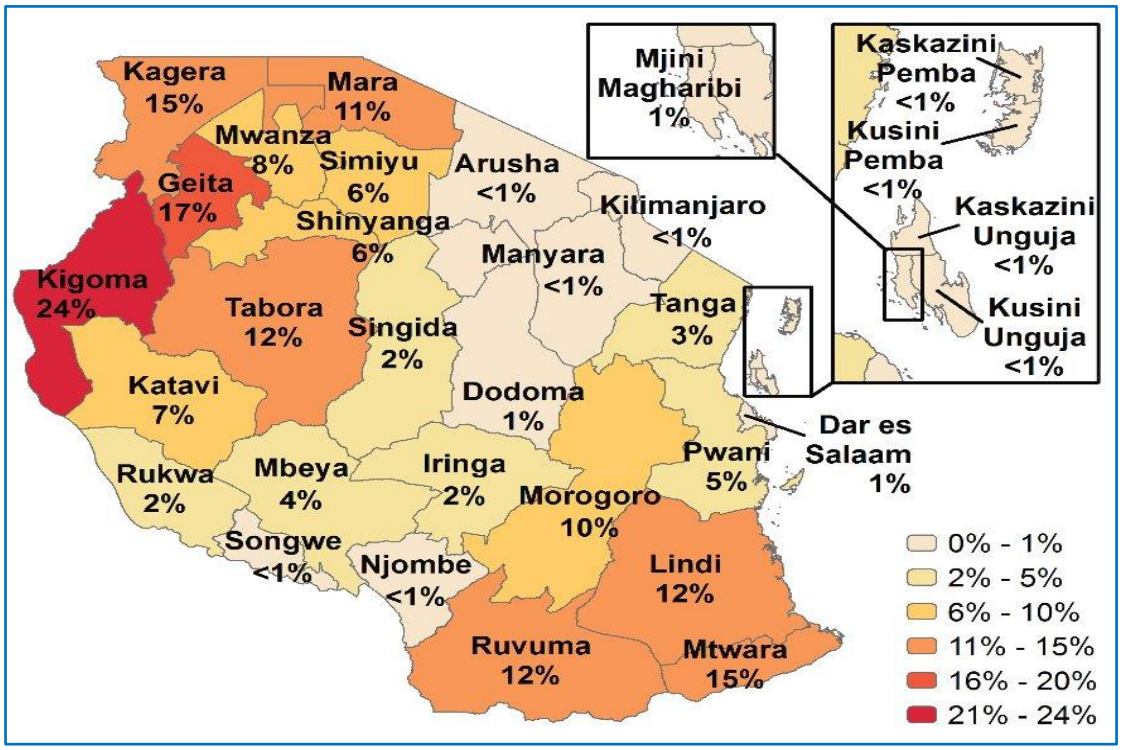

Figure 3. Tanzania map showing the pprevalence of malaria among children under five years of age by Region. Adapted from Tanzania Malaria Indicator Survey report (2018) [35].

1.4 MALARIA IN PREGNANCY

Pregnant women have increased risk of malaria infection than women who are not pregnant. Usually, adults from endemic areas have acquired immunity to malaria due to prior exposure over the life course. However, the high probability of malaria infection in women during pregnancy might be explained by two mechanisms. The first mechanism involves immune modulation which occurs during pregnancy. Usually, during pregnancy cortisol hormone levels are increased while the levels of prolactin hormone decrease. The increased cortisol hormone and the decreased prolactin hormone cause non-specific immunosuppression [38-40]. Additionally, there is temporary impairment of cell mediated immune to support the development of placenta and the growing fetus [41]. Since cell mediated immune mechanisms are crucial in malaria protection, their suppression during pregnancy partly explains the vulnerability of pregnant women to malaria [42]. The preferential accumulation of infected erythrocytes to the placenta is the second mechanism for the high risk of malaria during pregnancy.

In moderate and high endemic areas, primigravida women have increased chances of malaria infection, severity and malaria associated poor birth outcomes than multigravida [43]. On the contrary, in low-endemic areas, because of low acquired immunity, all pregnant women regardless of parity are equally susceptible to malaria and malaria associated adverse birth outcomes. The increased susceptibility among primigravida in moderate to high transmission areas is due to low placental parasite specific immunity [44] which is parity dependent and protects pregnant women in the subsequent pregnancies [45].

1.4.1 The pathogenesis of Placental Malaria

Malaria associated adverse birth outcomes occur due to accumulation of malaria infected erythrocytes to the placenta. In pregnant women, *P. falciparum* malaria parasites express unique variant gene (*var2csa*) which codes for unique adhesive variant antigens on the surface of infected red blood cells [46]. These adhesive molecules are part of *Pf*EMP1family specifically binding to chondroitin sulfate A and mediate the accumulation of malaria infected erythrocytes to the placenta. These var2csa proteins are the main targets for vaccine development against placental malaria [47]. The accumulation of infected erythrocytes to the placenta, causes inflammatory response [48]. The placental inflammation result in histological changes including the deposition of malaria pigment, penetration of mononuclear cells, complement deposition, the trophoblast basement membrane thickening and syncytial knotting [49-51]. Placental histological changes lead to changes in placental angiogenesis which causes changes in the architecture of placental villous [52] and surface area for nutrient transfer [53-55]. In turn, utero-placental blood flow is impaired, leading to insufficiency nutrient transport across the placenta, causing intrauterine growth restriction [51,56,57] (**Figure 4**).

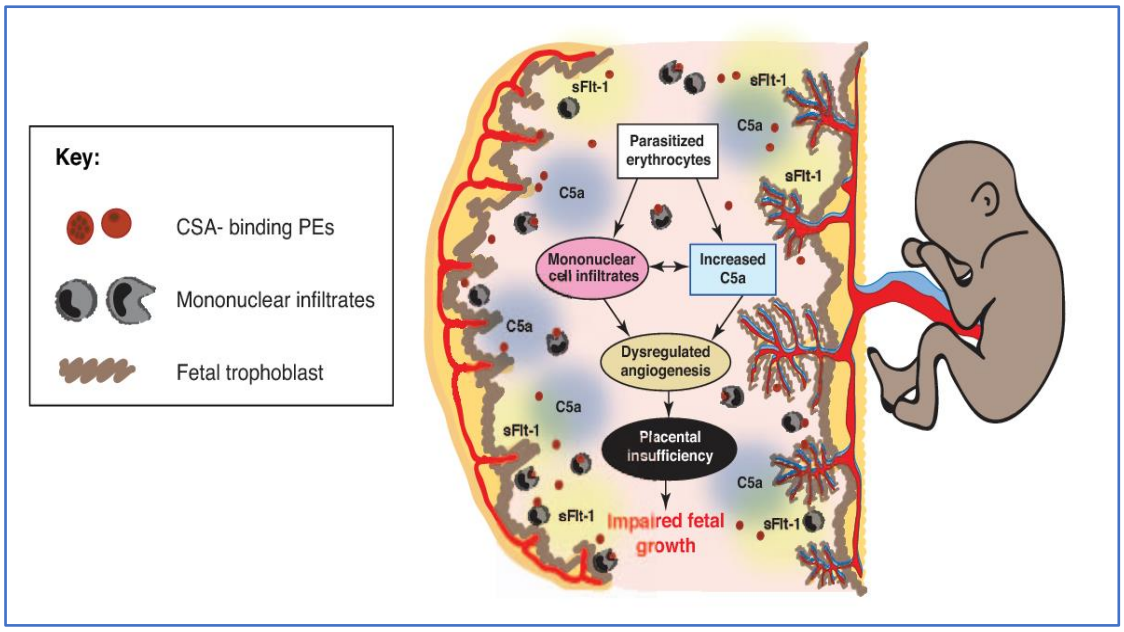

Figure 4. Scheme representing the pathogenesis of placental malaria. Abbreviations: CSA, chondroitin sulfate A; C5a, complement component 5a; PEs, parasitized erythrocytes; sFlt-1, soluble fms-like tyrosine kinase-1. Adapted from Conroy AL. et al. (2019) [58].

1.4.2 Maternal effects associated with Malaria in Pregnancy

Infection with malaria during pregnancy causes maternal illness associated with malaria fever, chills, and general body weakness. On rare occasions, severe malaria during pregnancy may occur and is usually associated with organ damages and a high risk of maternal mortality [59,60]. In sub-Saharan Africa, malaria in pregnancy is the main contributor of maternal anemia. Maternal anemia during pregnancy is an indirect cause of malaria-associated maternal death [61]. *Plasmodium falciparum* causes anemia by various mechanisms including hemolysis, increased splenic clearance of both infected and non-infected erythrocytes, and reduced erythrocytes production [62,63].

On the other hand, a study from Malawi estimated that, a single malaria episode may cost beyond a week's worth of income for families as direct and indirect economic effects [64]. Since women largely contribute to the family income, malaria in pregnancy may impose most families and communities in sub-Saharan Africa to the poverty cycle.

1.4.3 Fetal effects associated with Malaria in Pregnancy

Malaria-associated fetal growth restriction increases the risk of having LBW new-born. The risk of malaria-associated LBW increase when the infection is repeated during pregnancy [65]. In addition, the risk of having LBW is higher for women with placental infection compared to women with only peripheral blood parasitemia [66]. More than 800,000 LBW infants in sub-Saharan Africa were associated with malaria exposure during pregnancy in 2018 and 2019 [29,30]. The model estimates that infants born with LBW have a three-fold risk of mortality than infants born with normal birth weight [67,68]. Moreover, LBW infants

have a high risk of poor neurodevelopment and lower Intelligence quotient (IQ) compared to infants born with normal birth weight [69].

On the other hand, malaria in pregnancy is one of the substantial causes of stillbirth in Africa. Essentially, the risk of malaria-associated stillbirth is twice higher in low to moderate endemic areas compared to high endemic areas [70]. A systematic review estimates that, about 12-20% of stillbirths occurring in sub-Saharan Africa are attributed to malaria in pregnancy [70]. Additionally, malaria in pregnancy is also associated with preterm birth. In East Africa, a systematic review reported that pregnant women infected with malaria are three times more likely to have preterm birth than pregnant women without malaria [71].

1.4.4 Infants' effects associated with Malaria in Pregnancy

Maternal malaria infection may be the cause of congenital malaria [72] and early malaria infection in infants [73,74]. The association of malaria in pregnancy and the increased risk of infancy malaria may be explained by two hypotheses. Firstly, histological changes happening during placental infection may interfere with the passage of maternal antibodies to the offspring [75]. This may result in a compromised fetus and infants' immunity, thus increasing the risk of malaria infections in early childhood. Secondly, when the fetus is exposed to malaria in utero, it induces the development of regulatory T-cells (Treg), which cause tolerance of fetal immunity to malaria antigens that persevere to infancy [76]. Additionally, malaria in pregnancy increases the risk of infant anemia [77]. On the other hand, exposure to malaria in pregnancy was associated with less height and weight gain during the first year of life [78,79].

1.5 PREVENTION OF MALARIA IN PREGNANCY

To minimize the susceptibility of malaria among pregnant women, the WHO approved a package of preventive strategies among pregnant women residing in endemic areas. The package consists of ‘‘timely diagnosis and effective treatment of symptomatic malaria, intermittent preventive therapy during pregnancy (IPTp) with a drug sulfadoxine-pyrimethamine (SP), utilization of bed nets treated with insecticide (ITNs) and spraying indoor residuals’’ [80]. A systematic review of data from 32 countries in Africa indicated that the use of malaria prevention strategies significantly reduced LBW and infant mortality [81]. These strategies have been adopted and implemented by endemic countries in Africa including Tanzania [82].

1.5.1 Insecticide treated bed nets and residual spraying

Bed nets impregnated with the long-lasting insecticide, commonly pyrethroids, are known as insecticide-treated bed nets (ITNs). The recommendation for using ITNs as an extensive control strategy for prevention of malaria came forth in 1999 [30]. In the year 2000, the WHO approved the first long lasting ITNs for malaria prevention [30]. Currently, ITNs are extensively implemented for the control of malaria in endemic areas largely in Africa.

Between 2000 and 2015, the use of ITNs have prevented 68% of malaria cases in Africa [83]. Systematic review estimate that, the use of ITNs in pregnancy substantially reduce the risk of LBW, miscarriages, stillbirths and placental parasitemia [84,85]. Despite the demonstrated efficacy of ITNs in sub-Saharan Africa, there is slow coverage of INTs. For instance, the overall coverage of INTs was 61% in 2018 [29], similar to the coverage in 2015 [27]. In 2019, the estimated coverage of ITNs ownership in sub-Saharan Africa was 68% [30].

In Tanzania, according to malaria control policy, all pregnant women receive ITNs through antenatal care (ANC) and expanded program for immunization [86]. The coverage of ITNs ownership increased to 78% in 2018 [87]. However, low utilization of ITNs among pregnant women could be a challenge for malaria control among pregnant women. For instance, according to the national malaria indicator survey, the utilization of ITNs among pregnant women in Tanzania has droped from 75% in 2010 to 51% in 2017% [34,35]. On the other hand, a systematic review reported a rapid increase in mosquitoes' resistance to insecticides in Tanzania which threats the future performance of INTs [88]. Similarly, an increase in mosquitoes' resistance to insecticides has been reported from other countries in Africa [89]. A recent WHO recommendation indicates that bed nets may be treated with both pyrethroids and an added synergist insecticides like piperonyl butoxide in areas with reported mosquito resistance to pyrethroids [90].

1.5.2 **Intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP)**

1.5.2.1 Sulfadoxine-pyrimethamine (SP) and its mechanism of action

Due to widespread resistance of *P. falciparum*, SP was removed as the treatment regimen and restricted for IPTp. SP act through the inhibition of enzymes which catalyze crucial consecutive steps in the synthesis of folate products. Sulfadoxine is an analogue of *p*-amino benzoic acid which inhibit dihydropteroate synthase (DHPS) enzyme, a crucial step in the parasites' folate synthesis [91]. On the other hand, pyrimethamine inhibit competitively dihydrofolate reductase (DHFR) a key enzyme for the production of tetrahydrofolate, which is an important cofactor required in the biosynthesis of parasites nucleotides and proteins [92]. The fact that Mammalian cells acquire folate derivatives from dietary intake as they don't synthesize folates *de novo*, explain the selective activity of SP. Inhibition of DHPS and DHFR is synergistically important, leading to depletion of the important cofactors, which interrupts the synthesis of nucleotides and proteins, thus killing the parasite.

1.5.2.2 The IPTp-SP policy

In the year 1998, the WHO recommended IPTp as one of the control strategies for prevention of malaria during pregnancy [30]. IPTp-SP involves the administration of a single dose of SP at each monthly ANC beginning early second trimester till delivery. The IPTp policy changed overtime depending of research based evidence. Currently, according to the WHO policy, three and above doses of IPTp-SP given at least one-month interval are

considered as an optimal coverage [93]. Since the change of IPTp-SP policy to three and above as an optimal strategy, its coverage increased rapidly to 31% in 2015 [27]. Conversely, the uptake of optimal IPTp-SP (≥3 SP) in sub-Saharan Africa has slowed down since 2015. For instance, in 2017, the coverage of three and above doses of IPTp-SP in sub-Saharan Africa was 26% [28] and increased to 31% in 2018 [29], which was similar to the coverage in 2015 [27]. In 2019, the optimal IPTp-SP coverage increased to 34% [30].

A systematic review indicated that most countries in sub-Saharan Africa are far away from the 80% target coverage of optimal IPTp-SP [94]. Besides, studies reported different coverages from different countries in sub-Saharan Africa. For instance, a study from Ghana reported 63% coverage of optimal IPTp-SP [95]. In Benin, a study reported that 34% of pregnant women received optimal IPTp-SP [96]. The coverage of optimal IPTp-SP in Tanzania was 26% in 2017 according to malaria indicator survey [35].

Evidence from the literature indicates that optimal IPTp-SP (three and above SP doses) improve birth weight [97-100]. A previous study reported no association between optimal IPTp-SP and LBW [101]. On the other hand, the efficacy of optimal IPTp-SP (three and above SP doses) on malaria in pregnancy is controversial. While some studies indicate that receiving three and above doses of IPTp-SP is associated with protective effect on malaria in pregnancy [102,103], others reported that three and above doses of IPTp-SP does not protect malaria in pregnancy more than the lower doses of IPTp-SP [104-106]. The limited and inconsistent data on the efficacy of IPTp-SP, suggest the need to evaluate its performance regularly in order to provide updated information.

1.5.3 Effective malaria case management

Effective malaria case diagnosis and treatment during pregnancy is one of the malaria control strategies implemented. Usually, malaria rapid diagnostic test (mRDTs) and microscopy are used for routine diagnosis of malaria in sub-Saharan Africa. Symptomatic cases of malaria in pregnancy can be easily diagnosed using the standard methods and treated. Artemisinin-based combination therapies (ACTs) are used to treat uncomplicated malaria from the second trimester while oral quinine plus clindamycin are used during the first trimester as recommended by the WHO [107]. However, recent evidence suggests that ACTs could be safe for treating uncomplicated malaria in the first trimester [108]. ACTs have demonstrated high cure rates for the treatment of malaria during pregnancy [109]. Immediate treatment of malaria prevents chronic placental malaria and improves birth outcomes [110].

Nevertheless, most cases of malaria in pregnancy are asymptomatic. In asymptomatic malaria, there is usually low parasite density, thus most cases may be missed by the routine methods, microscopy, and mRDTs due to their limited sensitivity [111]. Therefore, asymptomatic malaria in pregnancy may remain untreated and cause adverse birth outcomes. On the other hand, asymptomatic malaria in pregnancy may serve as pool facilitating malaria transmission in endemic countries. A modeling analysis estimated that, in a non-pregnant population where 90% of people received mass drug administration for

malaria control, pregnant women may possibly contribute up to 23·9% of the new infections in the population [112]. In fact, the WHO has acknowledged asymptomatic malaria as one of the obstacles for successful control of malaria and requires control approaches to address asymptomatic malaria, especially in pregnant women [25]. However, for effective designing, planning and strengthening strategies for the control of asymptomatic malaria, data on the burden of asymptomatic malaria in pregnancy are needed. In sub-Saharan Africa, the burden of asymptomatic malaria in pregnancy could be high, but data are scarce. For instance, 35%, 50%, and 54% of pregnant women were found to have asymptomatic malaria at first ANC visit in Kenya, Uganda, and Malawi, respectively [113-115]. Given the limited data on the burden of asymptomatic malaria in sub-Saharan African countries, including Tanzania, research is needed to inform policy makers.

1.6 *P. FALCIPARUM* RESISTANCE TO SP AS A CHALLENGE FOR PREVENTION OF MALARIA IN PREGNANCY

Tracking of SP resistance is done through monitoring the key mutations in *P. falciparum dihydrofolate reductase* (*Pfdhfr*) and *dihydropteroate synthetase* (*Pfdhps*) genes. Various single nucleotide polymorphisms haplotypes in the *Pfdhps* and *Pfdhfr* genes cause *P. falciparum* resistance to sulfadoxine and pyrimethamine, respectively. Three mutations in *Pfdhfr* (N51I, C59R and S108N) and two mutations in *Pfdhps* (A437G and K540E), together form the quintuple haplotype. Quintuple haplotype is associated with high *P. falciparum* resistance to SP in East Africa [116,117]. An extra mutation (A581G) in the *Pfdhps* genes resulted in a highly resistant combination known as sextuple haplotype [118]. To evaluate the effect of SP resistance on the efficacy of IPTp-SP, the prevalence of *Pfdhps* K540E and *Pfdhps* A581G within the population represent quintuple and sextuple haplotypes, respectively.

In sub-Saharan Africa, the increasing prevalence of *P. falciparum* resistance to SP poses threats to the efficiency of IPTp-SP. In a multi-country study, the efficiency of SP to clear existing parasitemia and protect pregnant women against new malaria infections was found to decrease with the increasing prevalence of *Pfdhps* K540E mutation [119]. Equally, the effectiveness of IPTp-SP to prevent LBW was found to be lower in areas with a high population prevalence of *Pfdhps* K540E mutation [120]. The sub-optimal activity of SP to clear parasites may be one of the reasons for sustainable malaria transmission in sub-Saharan Africa. This might be explained by the fact that asexual parasites not cleared by SP could differentiate into sexual forms and increase peripheral gametocytemia [121], which are the transmittable stages of *P. falciparum* to the mosquito. In fact, some studies have reported that women who received IPTp-SP had significantly higher gametocytemia at delivery than those who did not receive IPTp-SP [122,123].

On the other hand, a systematic review reported that 10% population prevalence of *Pfdhps* A581G mutation could indicate the boundary for IPTp-SP efficacy above which it is no longer effective [124]. The WHO suggests stopping IPTp-SP in areas where the population prevalence of *Pfdhps* K540E mutation is greater than 95%, and the prevalence of *Pfdhps* A581G mutation is greater than 10%, since the efficacy of IPTp-SP is likely to be lost [93].

Various studies have reported diminishing efficacy of IPTp-SP in areas with a higher prevalence of A581G mutation. For example, a study from an area where the prevalence of *Pfdhps* A581G is 36% in Uganda reported that IPTp-SP efficacy on malaria and LBW was not found [125]. Similarly, studies from northeast Tanzania, where the prevalence of *Pfdhps* A581G mutation is high [126], reported loss of IPTp-SP efficacy [101,127]. Eventhough the population prevalence of *Pfdhps* A581G mutation is below 10%, infection with parasites harbouring *Pfdhps* A581G are associated with significantly lower birth weights among pregnant women receiving IPTp-SP [128]. Besides, a study from the Democratic Republic of Congo reported 47% population prevalence of A581G [129]. Similarly, the prevalence of *Pfdhps* K540E in Tanzania is 90.4% [130] close to saturation (>95%). Given the adverse effects of malaria during pregnancy and the increasing resistance of *P. falciparum* to SP, there is an urgent need to explore alternative strategies for effective control of malaria among pregnant women.

1.7 ALTERNATIVE STRATEGIES EVALUATED FOR PREVENTION OF MALARIA IN PREGNANCY IN SUB-SAHARAN AFRICA

In sub-Saharan Africa, extensive *P. falciparum* resistance to SP compelled the need for alternative strategies to prevent malaria in pregnancy. Various antimalarial drug regimens were assessed in the search for an alternative regimen for IPTp. In Ghana, amodiaquine or addition of amodiaquine to SP for IPTp was investigated against the standard IPTp-SP [131]. However, IPTp with amodiaquine or amodiaquine-SP was not tolerated and was not superior to the standard IPTp-SP. Similarly, a multicentre study reported that mefloquine was poorly tolerated and failed to replace SP for IPTp [132]. In addition, a combination of azithromycin and chloroquine evaluated in a multicentre study was less tolerated and did not have superior effects to the standard IPTp-SP [133]. On the other hand, an addition of two doses of azithromycin to monthly IPTp-SP in Malawi was found to improve birth weight [134] but not superior to IPTp-SP for malaria prevention [135]. Artemisinin-based combination therapies (ACTs) were used in studies to evaluate intermittent screening and treatment of asymptomatic pregnant women (ISTp) during ANC visits as an alternative strategy. However, results regarding the efficiency of ISTp are contradicting. Essentially, a multicentre study in west Africa reported that ISTp using artemether-lumefantrine (AL) was non-inferior to IPTp-SP [136]. On the contrary, a trial in Nigeria reported that ISTp-AL was superior to IPTp-SP [137]. On the other hand, two studies in high malaria transmission areas consistently reported that ISTp was inferior compared to IPTp-SP. One study from Malawi reported that ISTp using dihydroartemisinin-piperaquine (DHP) was significantly associated with a high prevalence of malaria at delivery than IPTp-SP [115]. Similar findings were reported by a trial done in Kenya [113]. Another multicentre trial in West Africa investigated whether adding regular screening and treatment of malaria with AL in the community to the IPTp-SP would improve maternal and infant health [138]. However, this approach did not reduce malaria in pregnancy significantly as compared to IPTp-SP alone. Based on the limited sensitivity of malaria rapid diagnostic tests currently used, the WHO concluded that ISTp should not be an alternative choice [139].

1.7.1 Optimization of artemisinin-based combination therapy (ACT) for prevention of malaria in pregnancy

In sub-Saharan Africa, ACTs are the first-line regimens for the treatment of malaria since 2004 [107]. The safety and efficacy of ACTs for treating uncomplicated malaria from the second trimester is well established [140-142]. However, the fact that most malaria cases during pregnancy do not present with clinical symptoms warrant the need to further explore ACTs as alternative regimens for IPTp. This need is supported by the malaria GTS 2016-2030 which recommends the optimization of existing strategies towards malaria elimination [25]. Among the available ACTs, DHP provides the most favourable features for this treatment approach. The combination of DHP is given once a day, therefore less compliance problems compared to other ACTs with more than once dosing per day. DHP is also well-tolerated, with the longest half-life providing sufficient post-treatment malaria prevention [143] which is needed for the IPTp regimen.

In east Africa, trials indicated that the combination of DHP could serve as an alternative regimen to SP for IPTp. A trial in Kenya evaluated IPTp with DHP versus the standard IPTp-SP and reported a significant reduction in parasitemia and malaria outcomes associated with IPTp-DHP when compared with the standard IPTp-SP [113]. Similarly, a trial from Uganda reported significant protection against malaria and parasitemia during pregnancy associated with IPTp-DHP compared to IPTp-SP [114]. Another trial from Uganda reported the safety of IPTp-DH given monthly and superior effects on parasitemia and placental malaria but not on negative birth outcomes [144]. However, the trials from Uganda included pregnant women with gestation ages between 16 to 20 weeks. In addition, the trials were conducted in areas with high malaria transmission intensities. Similarly, the trial from Kenya was done in a setting with high malaria transmission but compared three doses of IPTp-DHP or monthly IPTp-DHP versus IPTp-SP in women on their second and third trimesters. Bearing the influence of malaria transmission intensity on the impact of IPTp, it was not known whether the findings could be generalized to areas of moderate malaria transmission intensities. Furthermore, gestational age below 20 weeks is usually earlier than the really gestational ages at first ANC visits in most sub-Saharan African countries, including Tanzania. Therefore, more research was needed to be done in real-world settings and from geographical areas with moderate malaria transmission intensity in order to give more evidence [145].

1.8 THE PROPOSED DHP MECHANISM OF ACTION

The potent short-acting dihydroartemisinin ensures quick reduction in parasite population, leaving low parasite density. The long-acting piperaquine ensures sustained removal of the remained parasites and provide prophylaxis against new infection. The anti-malarial mechanism of action for dihydroartemisinin is hypothesized to be exerted by its endoperoxide bridge. The endoperoxide-bridge causes free-radicals which damage the parasite membrane systems through alkylating and oxidizing proteins and lipids in parasitized erythrocytes leading to parasite death [146,147]. On the other hand, the exact mechanism of action for piperaquine is not known. It is postulated that piperaquine works in

a similar mechanism like chloroquine where it accumulates in the digestive vacuole of matured asexual blood-stage trophozoites and inhibit haem-digestion. Normally, parasites in red blood cells obtain nutrients by breaking down hemoglobin into amino acids [148]. During this process, toxic by-products known as haematin is produced [149]. The parasite has a mechanism to detoxify haematin through polymerization to form non-toxic crystal structures known as hemozoin or malaria pigment [150]. Thus, the binding of piperaquine in heme hinders the polymerization resulting in the accumulation of toxic haematin, which destroys the parasite [147,151].

1.9 PIPERAQUINE PHARMACOGENETICS AND PHARMACOKINETICS

The physiological alterations which occur in pregnancy could affect the pharmacokinetics of various drugs in different ways [152]. For instance, increased levels of cortisol and estrogen hormones during pregnancy are associated with increased expression of drug-metabolizing enzymes and transport proteins [153] which could affect drug absorption and clearance. In addition, increased body fluids might affect drug volume of distribution. However, most studies reported that pregnancy could not significantly affect the overall exposure of piperaquine plasma level [154-156] when used for the treatment of uncomplicated malaria. On the other hand, some studies observed a decrease in piperaquine terminal elimination half-life but not the overall piperaquine exposure in pregnant women compared to non-pregnant counterparts [155,157]. The pregnancy-associated reduced terminal elimination half-life might affect the malaria prophylactic effect of DHP especially when used for IPTp. Nevertheless, the impact of piperaquine pharmacokinetics on the treatment outcomes during IPTp with DHP was not investigated.

Evaluating piperaquine pharmacokinetics in the context of IPTp is important for dosing optimization. Few studies have reported the pharmacokinetics of piperaquine in IPTp regimen. One study from Uganda conducted during IPTp with DHP, estimated the trough plasma piperaquine concentration of 10.3 ng/mL and 13.9 ng/mL to provided 95% and 99% protection against malaria, respectively [158]. Based on these targets, a recent study reported that, more than 90% of women who received IPTp-DHP in Kenya and Indonesia have attained the target trough concentration (10.3 ng/mL) after three doses of monthly IPTp-DHP [159]. Another study reported 72% higher piperaquine clearance in pregnant women than postpartum women [157]. For antimalarial drugs with a long elimination half-life, the plasma concentration at day 7 is well correlated with the area under the concentration curve (AUC) and could be used to monitor treatment efficacy [160]. In IPTp regimen, data regarding day-7 piperaquine pharmacokinetics are not available in the literature. It was therefore not known whether day-7 piperaquine concentration could also be used to evaluate the effectiveness of IPTp with DHP.

On the other hand, pharmacogenetic variations could significantly affect the pharmacokinetic exposure of anti-malaria drugs. Single nucleotide polymorphism occurring on genes coding for drug-metabolizing and transporter proteins could reduce, increase or diminish metabolism of drugs [161]. Despite this fact, the influence of genetic polymorphism on piperaquine is not well understood. One study evaluated the effect of pharmacogenetics

on piperaquine pharmacokinetics in a small sample size for the treatment of uncomplicated malaria in Cambodia reported no significant effects [162]. However, different ethnic groups have different distributions of gene alleles coding for CYP enzymes. In addition, there are no data on the effects of pharmacogenetics on plasma piperaquine level when used for IPTp. Given the proven superior malaria protective effect of IPTp-DHP compared to IPTp-SP, data of pharmacogenetics on piperaquine plasma concentration in IPTp is importantly needed to inform policy decisions.

2 AIMS OF THE RESEARCH

2.1 GENERAL OBJECTIVE

Generally, the PhD project aimed at optimizing the intermittent preventive therapy for prevention of malaria and negative birth outcomes among HIV uninfected pregnant women in Tanzania. The focus was to provide an insight on the burden of asymptomatic parasitemia before initiating IPTp, evaluate the effectiveness of monthly IPTp-DHP as compared to the current standard of care (IPTp-SP), and to explore the pharmacogenetics and pharmacokinetics of piperaquine in relation to treatment outcome during IPTp.

2.2 SPECIFIC OBJECTIVES

Paper I To investigate the prevalence and correlates of asymptomatic malaria and anemia at the first antenatal care visit before initiating IPTp among HIV uninfected pregnant women in Tanzania.

Paper II To evaluate the effectiveness of the current standard of care (IPTp-SP) for prevention of malaria during pregnancy and adverse birth outcomes among HIV uninfected pregnant women in Tanzania.

Paper III To compare the effectiveness of IPTp-DHP versus the standard IPTp-SP for prevention of malaria in pregnancy and adverse birth outcomes among HIV uninfected pregnant women in Tanzania.

Paper IV To explore the pharmacogenetics and day 7 pharmacokinetics of piperaquine component in DHP, and their relevance on treatment outcome among HIV uninfected pregnant women received monthly IPTp-DHP in Tanzania.

3 METHODOLOGICAL CONSIDERATIONS

3.1 STUDY DESIGN AND TARGET POPULATION

As shown in **Figure 5**, all four sub-studies targeted pregnant women at their first ANC visit. Sub-study one used cross-sectional design (**Paper I**). For sub-study three (**Paper III**), we used a superiority randomized controlled trial design. Sub-study two (**Paper II**) was a prospective observational study nested in the randomized controlled trial. Similarly, sub-study four (**Paper IV**) was a nested pharmacogenetics and pharmacokinetics study in a randomized controlled trial.

A set of inclusion and exclusion criteria were used for each respective sub-study. We determined trimester at enrollment by using the last Menstrual Period, and fundal height according to the national standard ANC guidelines [163]. **Paper I** included pregnant women both on their first, second and third trimesters. On the other hand, Papers II, III and IV included pregnant women only on their second and third trimesters. This is because the study drugs used for IPTp (Papers **II** and **III**) are only recommended to commence from the second trimester onwards [107]. At enrollment, participants were screened for malaria using mRDT and PCR. For **paper I**, both pregnant women with asymptomatic malaria and malaria-free were included. On the other hand, women with patent malaria (detected by mRDT) were excluded for Papers **II**, **III** and **IV**. We excluded pregnant women with patent malaria because they received treatment with a drug other than the study drugs (artemether-lumefantrine), which could affect the study outcomes. In the four studies, we excluded pregnant women with a history of malaria and treatment for the past one month. This is because the tests we used to screen for malaria (mRDT) at enrollment could still give false-positive results within the period of one month since the diagnosis as they depend on antigen-antibody reaction. HIV-infected women were also excluded. Furthermore, women with severe anemia were excluded and referred for further management.

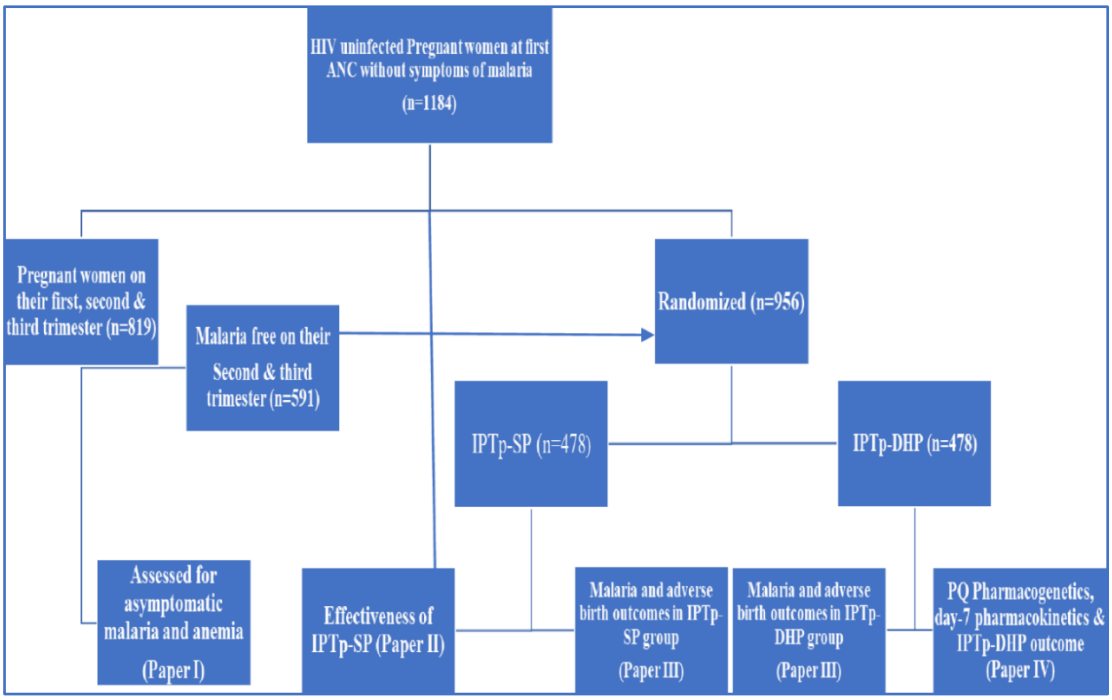

Figure 5. Summary of the participants' population recruited for all the four sub-studies.

3.2 PARTICIPANT RECRUITMENT AND BASELINE DATA COLLECTION PROCEDURES

In all four sub-studies, we used standard Case Report Forms (CRF) for data collection. We collected information regarding sociodemographic, gravidity, parity, gestational age, ITN use, medication use, maternal age, and level of education at enrollment in order to assess their impacts on our study outcomes. Following standard procedures, weight and height were measured using a digital weighing scale in kilograms (nearest 0.1kg) and a potable wooden scale in centimeters (nearest 0.1cm), respectively. Body temperature was determined from the maternal armpit using a digital thermometer and expressed in degree Celsius (^{o}C). A temperature of $\geq$37.5^{o}C was considered as fever.

3.3 RANDOMIZATION AND ADMINISTRATION OF STUDY DRUGS

For **paper III**, participants were randomly assigned (1:1) allocation to receive either IPTp-DHP or IPTp-SP using a computer-generated randomization list. Opaque, sealed, and sequentially numbered envelopes with blind treatment allocations were used to assign pregnant women to their respective study groups. In order to ensure adherence to allocations, we monitored regularly the sequential arrangement of envelopes. Participants in IPTp-DHP arm received a dose of three tablets containing dihydroartemisinin-piperaquine fixed combination (D-ARTEPP, Guilin Pharmaceutical Co. Ltd, China) once daily for three consecutive days. Each tablet contained 40 mg of dihydroartemisinin and 320 mg of piperaquine. The first dose was given as directly observed therapy (DOT) at the ANC. The remaining second and third doses were taken at home 24th and 48th hours after the first dose, respectively.

On the other hand, women in the standard group (IPTp-SP) received a single dose of three tablets, and each tablet contained 500 mg of sulfadoxine and 25 mg of pyrimethamine (Orodar, Elys Chemical Industries Ltd, Kenya) as DOT at the ANC. The Tanzania National Malaria Control Programme supplied SP tablets through the Medical Stores Department. In addition, all participants received mebendazole 500mg and a fixed combination containing ferrous sulphate (150mg) plus folic acid 0.5mg once daily for prevention of anemia during pregnancy according to ANC guidelines [163]. Tablets with folic acid more than 1.5mg were not supplied to the study participants because they might interfere with the IPTp-SP malaria protection effect [164,165].

3.4 PARTICIPANTS FOLLOW UP PROCEDURES

Participants in sub-study two, three, and four were scheduled monthly for follow-up visits and screened for malaria and anemia. In addition, they received their respective drugs on each monthly ANC visits until delivery. Women who visited study health facility out of their scheduled visits were screened for malaria and fever by the study clinicians. Furthermore, all participants were assessed on ITNs use at each scheduled ANC visit and followed till delivery.

For sub-study two and three, we recorded adverse birth outcomes from participants and newborns. To assess for low birth weight, study midwives weighed newborn babies on a digital scale and measured their weight to the nearest 10 g immediately after birth. Birth weights below 2500g was considered as LBW. In addition, study midwives examined newborn babies for congenital malformation within the first 24 hours of delivery. Also, we recorded any miscarriages (occurring below 27 weeks gestational age), still births (occurring ≥28 weeks gestational age), premature birth (occurring <37 weeks gestational age), and neonatal or maternal deaths. After delivery, we followed participants up to six weeks for the purpose of monitoring any congenital anomalies, maternal or neonatal deaths within that period.

3.5 PARTICIPANTS' MATERIALS AND BLOOD SAMPLE COLLECTION

We collected blood samples and placental tissue to evaluate the study outcomes. Blood samples were collected using standard routine procedures by experienced laboratory technicians. At enrollment, we collected 2ml of venous blood from participants on the IPTp-DHP arm for pharmacogenetics study (**Paper IV**). Similarly, pregnant women who received IPTp-DHP had a scheduled visit at day 7 after each monthly administration of study drugs, where 3ml of blood was collected for piperaquine pharmacokinetics (**Paper IV**). The whole blood was immediately centrifuged at 2000 × g for 10 minutes to obtain plasma. The obtained palsma was aliquoted into plastic cryo-vial and stored at -80 ^{o}C. We used plastic cryo-vial because piperaquine could readily adsorb to glass tubes due to its multiple nitrogen atoms and concsequently affect the plasma concentration. Moreover, we used day 7 single sampling for pharmacokinetics because it is well correlated with the area under the plasma concentration curve (AUC) and it is recommended for monitoring

the efficacy of antimalarial drugs with a long elimination half-life, including piperaquine [166].

During each scheduled monthly ANC visit, peripheral finger-prick blood was collected for determination of hemoglobin concentration and parasitemia using mRDT and PCR (Papers **II, III** and **IV**).

At delivery, blood samples were collected for assessing malaria and anemia outcomes (Papers **II**, **III** and **IV**). Maternal venous blood, placental blood, and cord blood were collected in EDTA tubes for malaria screening. For further screening of malaria using PCR, we collected dried blood samples (DBS) in Whatman filter paper (Whatman, Inc. NJ, USA) from finger prick at enrollment and at each scheduled ANC visit and from maternal venous blood, cord blood and placental blood at delivery. To detect histopathological placental malaria for sub-studies **II, III** and **IV**, a section of placenta biopsy (approximately 2 cm^3) was collected from the maternal side and fixed in 10% buffered formalin.

3.6 DIAGNOSIS OF ANEMIA

Maternal anemia was monitored as a secondary outcome for sub-studies I, II and III whereas fetal anemia was a secondary outcome for sub-studies **II** and **III**. We determined Hemoglobin concentration from cord blood (fetal anemia) and maternal blood (maternal anemia). To determine anemia, we measured Hb concentration (g/dl) using a digital HemoCue Hb 201+ analyzer (HemoCue AB, Angelholm, Sweden). Briefly, a drop of blood was placed on the test strip, and the strip was inserted into the digital machine for reading. Maternal anemia was confirmed when maternal Hb was <11g/dL. Anemia severity was defined as Mild (10–10.9 g/dL), moderate (7–9.9 g/dL), and severe (<7 g/dL) according to WHO classification of anemia [167]. Fetal anemia was confirmed when cord blood Hb is <12.5 g/dL [168].

3.7 DETECTION OF MALARIA

Malaria was the primary outcome measure for sub-studies I, II and III and a secondary outcome measure for sub-study IV. We used a combination of diagnostic techniques to screen for malaria in order to ensure precise detection. This is because we enrolled pregnant women who were asymptomatic and were likely to have low densities of parasitemia, which may not be detected by mRDTs and microscopy with limited sensitivity. For sub-study I, both mRDTs and PCR was used to screening for malaria. For sub-studies II, III and IV we used mRDTs, microscopy, PCR and histopathology for malaria detection. Histopathology is a gold-standard method for screening malaria in placental tissue with good sensitivity. In addition, PCR has good sensitivity to detect parasitemia as compared to mRDTs and microscopy.

3.7.1 Detection of malaria by mRDT and microscopy

We used mRDTs, which are the current standard of care for the diagnosis of malaria in Tanzania. Malaria RDTs (Care start, ACCESS BIO Somerset, NJ, USA) were performed according to the manufacturer's instructions. Briefly, about two drops of blood were poured followed by wash buffer and examined after 20 minutes. The mRDTs detect two parasites' antigens namely; histidine-rich protein 2 (HRP2), specifically for *P. falciparum,* and Plasmodium lactate dehydrogenase (pLDH) an antigen marker for *P. ovale, P. vivax, and P. malariae*. Positive test was confirmed by a visible red-colored line which is a result of antigen-antibody complex occurring through the interaction between the parasites antigen in the blood sample and the monoclonal antibody on the test strip.

On the other hand, microscopy, which is gold standard diagnostic method to screen for malaria parasites, was used to confirm symptomatic malaria during pregnancy and parasitemia at delivery for sub-studies **II, III** and **IV**. Briefly thick blood smears on microscopy glass slides were prepared, stained with 2% Giemsa, dried and examined with a 100X oil immersion objective by experienced laboratory technicians. The absence of asexual parasites and/or gametocytes on the thick blood smear examined at 100 high-power fields was considered to be negative.

3.7.2 Screening of malaria by histopathology

Placental malaria was detected from placental tissue according to the method previously described [169,170]. Briefly, placenta tissue was dehydrated using Leica tissue processor (Leica Biosystems, Wetzlar, Germany) with ethanol at different concentrations serially, starting with 70%, 80%, 95%, and absolute ethanol, respectively. Then, ethanol was removed from the tissue specimens by xylene and embedded in paraffin wax. Microtome blade (Leica Biosystems, Wetzlar, Germany) was used for sectioning embedded tissue specimens into slides which were then stained with hematoxylin-eosin and Giemsa. Two slides were prepared for each placental tissue sample and read under microscopy in duplicate by two independent readers. Discrepant readings between the two microscopists were taken to an independent third reader, and conclusive results was based on two readers. Evidence for positive active placental malaria infection was confirmed when malaria parasites or parasites and pigments were observed in placental tissue. On the other hand, the detection of malaria pigments only in the intervillous fibrin and macrophages within the placental tissue was conclude as past placental malaria infection.

3.7.3 Detection of malaria by Real-Time-PCR

Genomic DNA was isolated from dried blood spots (DBS) using QIAamp DNA blood micro kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. We used 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) to screen for Plasmodium infection (*P. falciparum, P. vivax, P. ovale, and P. malariae*) earmarking the 18S rRNA gene using a previous method [171] with minor modification as described previously [172]. Briefly, each specific probe for species was labeled with a

distinguished fluorophore, and Mustang purple was used as a passive reference dye. Each PCR reaction contained a final volume of 15 μL. The volume included 7.5 μL of TaqMan multiplex master mix (Applied Biosystems), 0.3 μL (10 μmol/L) of each species-specific forward primers, 0.75 μL (10 μmol/L) of the reverse primer, 0.15 μL (10 μmol/L) of each species-specific probe, Mustang Purple, 1.95μL DNA/RNA-free water and 3 μL sample DNA. We used 45 PCR cycles to analyze the samples, beginning with 95 °C for 20 s, followed by thermal cycles of 95 °C for 3s, and of 60 °C for 20 s. We also used negative and species-specific positive standard controls on each reaction plate. Optimization of the assay was done to ensure that all the four species are detected simultaneously.

3.8 DNA EXTRACTION AND GENOTYPING

We extracted genomic DNA from whole-blood samples using QIAamp DNA Midi Kit (Qiagen GmbH, Hilden, Germany) as instructed by the manufacturer. Then, we genotyped for common functional variant alleles reported to be relevant in piperaquine metabolism [173]. Genotyping was done according to the method previously described [174]. In brief, we did genotyping using TaqMan drug metabolism genotyping assay reagents for allelic discrimination (Applied Biosystems Genotyping Assays). The reagents had the following ID numbers for each SNP: C_11711730_20 for *CYP3A4*1B (−392A>G*, rs2740574), C_26201809_30 for *CYP3A5*3* (*c.6986A4G*, rs776746), C_30203950_10 for *CYP3A5*6 (g.14690G4A*, rs10264272*)* and C_32287188_10 for *CYP3A5*7 (g.27131_27132*insT rs41303343). For *CYP2C8*, ID numbers were C_30634034_10 for *CYP2C8*2* (*g.11054A>T*, rs11572103), C_25625794_10 for *CYP2C8*3* (*c.416G>A*, rs11572080) and C_25761568_20 for *CYP2C8*4* (*c.792C >G*, rs1058930). We used 7500 Real-Time PCR system (Applied Biosystems) for genotyping. For each PCR reaction, the final volume was 10 μL, including 9 μl of TaqMan fast advanced master mix (Applied Biosystems, Waltham, MA, USA), DNA/RNA free water, TaqMan 20X drug metabolism genotyping assays mix (Applied Biosystems) and 1 μl genomic DNA. The PCR profile included an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read stage with 60 °C for 30 s.

3.9 PHARMACOKINETICS QUANTIFICATION OF PLASMA PIPERAQUINE

3.9.1 Materials

The following materials and chemicals with their procurement sources were used for plasma piperaquine quantification. Piperaquine Tetraphosphate and Piperaquine-d6 Tetraphosphate were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Acetonitrile (LC/MS grade) and methanol (LC/MS grade) were purchased from Fisher Scientific Co. (Beerse, Belgium). Triethyl ammonia (LC/MS grade) was purchased from Sigma-Aldrich (Missouri, USA). Formic acid (Optima™ LC/MS grade) was purchased from Fisher Scientific Co. (Brno-Černovice, Czech Republic). Ultrapure water was produced with an ELGA Maxima system from Ninolab (Stockholm, Sweden). Blank human plasma (K3EDTA added as an anticoagulant) was obtained from the

clinical Pharmacology laboratory at Karolinska University Hospital (Huddinge, Stockholm, Sweden).

3.9.2 Piperaquine quantitation method

We determined plasma piperaquine concentrations using Ultra high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method. We used a previously described method [175] with some minor modification. Briefly, we first prepared a diluent consisting of acetonitrile:water (1:9 v/v) and 0.5% formic acid. We used this diluet to prepare stock solutions for piperaquine reference standard and internal standard (piperaquine-d6). Then, we prepared standard samples for calibration (15.63, 62.5, 250, 1000 and 10000 ng/mL) using a serial dilution method with one batch of blank plasma. The standard samples at 31.25, 125, and 500 ng/mL were prepared with a different batch of blank plasma and were used as lower, middle and high quality control (QC) samples, respectively.

We used methanol (LC/MS grade) to precipitate plasma proteins. In brief, we added 50 µL of plasma sample and 50µL of internal standard piperaquine-d6 (100ng/mL, diluted in acetonitrile: water at a ratio of 1:9 and 0.5% formic acid) to 300 µL of methanol. The solution was briefly vortex-mixed and centrifuged at 25,000g for 5min. Then, we carefully transferred 100µL of the supernatant to a plastic 96-well plate placed on autosampler and 10 µL was injected to LC-MS/MS. Piperaquine could readily adsorb to glass tubes due to its multiple nitrogen atoms and this may consequently affect the assay. Thus, we used plastic Eppendorf tubes for sample preparation.

Furthermore, we used ACQUITY BEH C18 2.1 x 50mm, 1.8µm column (Waters, Milford, Massachusetts, USA) for chromatographic separation of analytes. Compounds were eluted with 0.1% triethyl ammonia in ultrapure water (solvent A) and 0.1% triethyl ammonia in acetonitrile (solvent B) at a flow rate of 0.6 mL/min. The analytes were eluted from the column using a linear gradient, starting at 40% solvent B (0 minute), isocratic hold for one minute (0-1 minute), then increased from 40% to 90% solvent B for two minutes (1-3 minutes), and then to 95% solvent B for 0.1 minute (3-3.1minutes), hold for one minute (3.1-4.1 minute), and then back to 40% solvent B (4.1-4.2 minutes). The total run time was 5 minutes, but the compounds were eluted after two minutes (**Figure 6**).

The analysis was set to also analyze QC samples after thirty clinical plasma samples to ensure the accuracy and precision of the assay. In total QC samples were run three times in each batch of 96 clinical samples. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 1.5 ng/mL and 15.63 ng/mL, respectively. The validation parameters were within the acceptable ranges according to FDA guidelines for method validation [176].

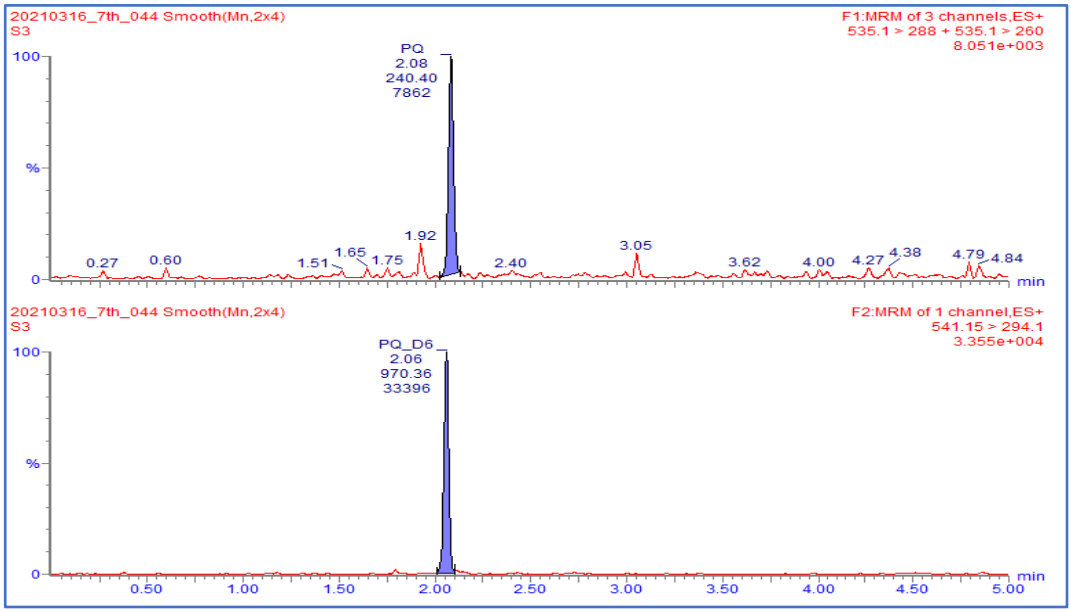

Figure 6. The Liquid Chromatography Mass Spectrometry Chromatogram of piperaquine and piperaquine-d6 (internal standard).

3.10 ETHICAL ASPECTS

All the four studies included in this thesis were conducted according to ethical principles complied with studies involving human subjects. Before participants' recruitment the protocol for all the sub-studies was reviewed and approved by the ethical review boards of the Muhimbili University of Health and Allied Sciences (2016- 06-07/AEC/Vol.XI/2) and the Tanzania National Institute for Medical Research (NIMR/HQ/R.8a/Vol.IX/2342). The clinical trial (**Paper III**) was registered with WHO-Pan African Clinical Trial Registry (PACTR201612001901313). All participants gave written informed consent. Participants who could not read and write gave thumb print signature to the consent form.

For all sub-studies blood samples were collected for screening of malaria and hemoglobin concentration. For sub-study **IV** blood samples were collected for pharmacogenetics and pharmacokinetics. The procedures for blood withdraw were done according to routine procedures by experienced and skilled laboratory technicians to minimize harms. In addition, we minimized the frequency of blood withdrawal to reduce pain experience among participants. For example, the single blood sample taken at enrollment was used for malaria screening, Hemoglobin determination and the rest was stored in -80 °C freezer for pharmacogenetics studies. For determination of plasma piperaquine concentration, we used single-point sampling at day 7 which is recommended by the WHO for monitoring exposure of antimalarial drugs with long elimination half-life [166].

In sub-studies **II, III** and **IV,** participants were followed until delivery. Scheduled visits were set according to the routine ANC visits to avoid unnecessary disturbance. Additionally, participants were supposed to make day-7 visits for assessment of adverse events and collection of blood samples for plasma piperaquine pharmacokinetics. In such

visits, pregnant women were compensated for their time and fare. The compensation was kept relatively reasonable to cover the time and fare to avoid coercion.

For sub-studies **II** and **III** participants received study drugs. The drugs used are part of the public health control strategies for the prevention and treatment of malaria in Tanzania recommended during pregnancy from the second trimester. We included pregnant women on their second and third trimesters, thus, unusual adverse drug events were not anticipated. Pregnant women were randomized; thus, the risk and benefits were fairly distributed among participants. For sub-study IV, whole blood and plasma samples were transported to Karolinska Institutet Sweden for pharmacogenetics and pharmacokinetics analysis, respectively. Participants agreed with sample transfer for this analysis. In addition, the analysis of samples in Sweden was approved by the Stockholm Ethics committee (Reference number=2020-00857). To ensure participants' confidentiality, all the personal information and clinical data collected were kept secret, and no names were used; instead, anonymous study identification numbers were used.

3.11 DATA MANAGEMENT AND STATISTICAL ANALYSIS

We employed experienced and trained clinicians and nurses for data collection. We used both paper and electronic CRF specifically created for this project (Census and Survey Processing system version 7, US Census Bureau, USA). To ensure quality control of samples, we consulted a senior laboratory scientist to assess 10% of randomly selected microscopy slides. In addition, we assured the quality and consistency of documentation whereby the PhD student cross-checked 10% of the CRF transferred from hard copies to the electronic CRF.

We used descriptive statistics such as percentages, mean (one standard deviation), median (range) to present baselined characteristics (Papers I, II, III and IV) and malaria outcomes collected at delivery (Papers II, III and IV). We presented repeated malaria and anemia outcomes collected at ANC during pregnancy as cumulative incidence or incidence rate with 95% confidence interval. We used Figures and Tables to present data in all the sub-studies.

3.11.1 Statistical tests

Chi square test or Fishers exact test were used to compare the prevalence of malaria and anemia (Papers I, II, and III**)**. The Shapiro–Wilk test was used to assess normality of continous data (Papers I, II, III and IV). Hosmer and Lemeshow test was used to assess the goodness of model fit in logistic resgression analysis. Cohen's kappa coefficient test was used to assess the agreement of methods used for screening malaria (Papers I and II). An Independent t test was used to compare the mean birth weights between newborns of mothers who received three and above IPTp-SP versus those who received less than three IPTp-SP (**Paper II**) and between those who received IPTp-DHP versus IPTp-SP (**Paper III**). Mann-Whitney U test was used to compare mean ranks of skewed baseline data between the treatment groups (Paper III). We also used paired-t-test to compare log

Piperaquine Plasma concentration between after receiving the 1st, 2nd, and 3rd IPTp doses. McNemar's test was used to compare proportions of anemia at enrollment and at delivery (Paper II). Poison regression model for count data was used to compare the incidence rate of parasitemia during pregnancy between the treatment groups (Paper III).

3.11.2 Models of analysis

Table 1 indicates different analysis models utilized for each individual sub-study. For each analysis model used, the multivariate analysis included variables with $p \leq 0.2$ in the univariate model. We used Cox Regression model to examine the predictors of parasitemia during pregnancy (Papers II and IV). We also used Kaplan Meir plot with Log Rank test to visualize graphically the significant factors associated with parasitemia during pregnancy overtime (Papers II and IV**)** as well as the risk of parasitemia during pregnancy over time between the treatment groups (Paper III). Logistic regression models were used to explore the factors associated with asymptomatic malaria and anemia (Paper I) and factors associated with malaria at delivery and adverse birth outcomes (Paper II and IV). Univariate ANOVA was used to compare mean day-7 piperaquine concentration between different genotypes (Paper IV). We also used Repeated measures ANOVA to compare between subject and within subject variations in day 7 piperaquine plasma concentration overtime (Paper IV). General Linear Model was used to explore factors associated with hemoglobin concentration (Paper I). We also used linear mixed model to explore factors associated with change in day-7 piperaquine concentration over time (Paper IV).

In **Paper III,** the Intention to treat (ITT) population which included all pregnant women allocated to treatment groups at enrollment was used as a primary analysis (Figure 1 of Paper III). Additionally, per-protocol population, which included pregnant women with collected primary outcome (histopathological placental malaria) at delivery, was used as a secondary supporting analysis (Paper III). In addition, the prevalence ratio was defined as the prevalence of an outcome in the intervention group (IPTp-DHP) divided by the prevalence in the standard group (IPTp-SP). Similarly, the incidence rate ratio was defined as the incidence measure in the intervention group (IPTp-DHP) divided by the incidence measure in the standard group (IPTp-SP). Then, the differences between the two groups were estimated by protective efficacy (PE) defined as **1-prevalence ratio** or **1-incidence rate ratio**. Furthermore, we did a secondary analysis for the outcomes between the two groups stratified by gravidity and excluding or including sub-patent parasitemia at enrollment (Paper III).

For data analysis, we used Statistical Package for Social Sciences (SPSS) software version 27 (Armonk, NY: IBM Corp). Also, we used Graph Pad Prism version 8.3 for Windows (Graph Pad, La Jolla, CA, USA) for graphical presentations. At 95% confidence level, p-value of < 0.05 was considered to indicate statistical significance.

Table 1: Different models of analysis used in different sub-studies

Type of Analysis	Paper I	Paper II	Paper III	Paper IV
Intention to treat			×	
Per protocol			×	
Logistic regression	×	×		×
GLM	×			
Univariate ANOVA				×
Repeated measure ANOVA				×
Kaplan Meir plot		×	×	×
Cox regression		×	×	×
Linear mixed model				×
Descriptive statistics	×	×	×	×

4 RESULTS AND DISCUSSION

From January 2017 to May 2019, a total of 1,184 pregnant women were recruited. To determine the burden of asymptomatic malaria and anemia, a total of 819 pregnant women on their first ANC visit were included (**Paper I**). Out of 819 women, 591 met inclusion criteria and enrolled for sub-studies **II** and **III**. In total, 956 pregnant women were (1:1) randomized to receive either monthly IPTp-SP (n=478) or IPTp-DHP (n=478) (**Paper III**).

To evaluate the effectiveness of the standard IPTp-SP, we prospectively followed pregnant women allocated to monthly IPTp-SP for parasitemia and anemia during pregnancy as well as malaria and negative birth outcomes at delivery (**Paper II**). Then, we compared IPTp outcomes between women allocated to IPTp-DHP versus those allocated to IPTp-SP (**Paper III**). In **Paper IV** we prospectively followed women allocated to IPTp-DHP (n=446) for piperaquine pharmacogenetics, monthly day 7 pharmacokinetics and their impact on IPTp-DHP outcomes.

4.1 ASYMPTOMATIC PARASITEMIA, ANEMIA AND THEIR CORRELATES AT FIRST ANC BEFORE INITIATING IPTp (PAPER I)

In paper I, we postulated that, several women initiating ANC might be asymptomatic but with parasitemia and associated anemia. In this study, we found 5.4 months as the median gestational age at first ANC indicating late initiation of ANC.

4.1.1 Asymptomatic parasitemia and anemia

We found that, 36.4% (95% CI=33.1 to 39.8; 298/819) of women had asymptomatic malaria associated with anemia (68.5%) at their first ANC visit. Malaria RDT detected 42.3% (126/298) of all asymptomatic malaria cases indicating the limited sensitivity of mRDT to detect asymptomatic parasitemia as compared to PCR.

To obtain the required sample size for this sub-study (n=819), we recruited women for a period of one year. Thus, we observed the prevalence of asymptomatic parasitemia at each month persisted above 25% throughout the year (Figure 4 of **Paper I**).

Our finding indicates that asymptomatic parasitemia and associated anemia at first ANC is common among pregnant women in Tanzania. This finding is comparable to several other studies conducted in sub-Saharan Africa [177-181]. Taken together, these data suggest that asymptomatic parasitemia during pregnancy is a public health problem that needs immediate attention. It may be surprising to observe such a high prevalence of asymptomatic parasitemia given the proved efficacy and high coverage of ITNs [35]. However, studies indicate the substantial change of *An. arabiensis* an outdoor biting mosquito from being rare to common [182,183]. The increase in the composition of outdoor biting mosquitoes may explain the observed prevalence of asymptomatic malaria. In addition, low utilization of ITNs could partly explain the observed finding. Outdoor biting mosquitoes does not only affect pregnant women but also the entire control

strategy for malaria, considering that key strategies in Africa (ITNs and indoor residual spray) mainly target indoor mosquito biting. Novel strategies especially targeting outdoor malaria vectors, are importantly needed.

To estimate the burden of asymptomatic malaria accurately, we used both mRDT and PCR. We found nearly half of asymptomatic malaria cases as patent infection (detected by mRDT), suggesting a high density of parasitemia among women. This finding also suggests that integrating screening with mRDT and treatment of positive cases with efficacious drugs at first ANC would benefit pregnant women, especially primigravida [184].

Asymptomatic malaria in pregnancy may serve as a parasite reservoir contributing to malaria transmission cycle. This thesis did not measure gametocytemia which is an infective stage to mosquito. However, a study from Malawi reported that 5% of pregnant women at first ANC had gametocytemia, suggesting that pregnant women could substantially contribute to malaria transmission [185]. Furthermore, it is estimated that pregnant women may contribute more than 20% of malaria transmission to the public in a population where 90% of people have received mass drug administration for malaria elimination [112]. We recommend regular revising and improving the control strategies for malaria in pregnancy. This could benefit the efforts for malaria elimination and contribute to the achievement of SDG 3.3, targeting to end among others the epidemics of malaria infection by 2030 [186].

4.1.2 Correlates of asymptomatic malaria and anemia

We further examined independent correlates of asymptomatic malaria and anemia using logistic regression model. We found that primigravida (p=0.005) and adolescent women (p=0.02) had significantly higher odds of asymptomatic malaria compared to multigravida and adult women, respectively (Table 2 of **Paper I**). Equally, asymptomatic malaria, maternal age, gravidity and gestational age were significant predictors of anemia (Table 3 of **Paper I**).

High burden of malaria in primigravida and adolescent women as compared to multigravida and adult women may be explained by parity and age-related immunity respectively as previously reported [187-190]. The correlation of asymptomatic malaria and anemia could explain the similar high burden of anemia in primigravida and adolescent pregnant women.

4.2 EFFECTIVENESS OF THE STANDARD IPTp-SP FOR PREVENTION OF MALARIA AND ADVERSE BIRTH OUTCOMES IN PREGNANT WOMEN (PAPER II)

In **paper II** we evaluated the effectiveness of the standard IPTp-SP to clear the observed sub-patent malaria (**Paper I**), prevent new infections and the consequent adverse birth outcomes among pregnant women. A cohort of 500 pregnant women was enrolled, administered monthly IPTp-SP and prospectively followed for IPTp outcomes till delivery. Baseline and follow-up characteristics are presented in Table 1 of **Paper II**. About three-quarters (256/417, 61.4%) of women received at least three doses (≥3 doses) of IPTp-SP which is considered as an optimal coverage. At delivery, 83% (417/500) of enrolled women had their primary outcome (histopathological placental malaria) collected (Figure 1 of **Paper II**).

4.2.1 Malaria and adverse birth outcomes during pregnancy and at delivery

To evaluate the effectiveness of IPTp-SP, we prospectively determined the incidence of symptomatic malaria during pregnancy, parasitemia and anemia during ANC visits. We also recorded adverse birth outcomes and screened for parasitemia and placental malaria at delivery. During the follow-up period, 2.8% (14/500) and 16% (80/500) of women receiving monthly IPTp-SP had symptomatic malaria and parasitemia respectively. Furthermore, about one fifth (20.9%, 87/417) of women had any parasitemia detected at delivery (Table 2 and Figure 2 of **Paper II**). In addition, 9.4% (39/417) of women had histopathological confirmed placental malaria among which 74% (29/39) was active placental infection (parasites) and 26% (10/39) was past infection (hemozoin pigments). The prevalence of malaria at delivery detected by different methods are presented in Table 2 of **Paper II**. The prevalence of composite adverse birth outcomes at delivery was 26.5% (114/430) among which 10.9% (46/423) of women had low birth weight newborns.

We found considerable burden of parasitemia during pregnancy and at delivery among women receiving monthly IPTp-SP. In this sub-study, we enrolled malaria-free (mRDT) women, thus IPTp-SP was expected to clear sub-patent malaria (not detected by mRDT but detected by PCR) and prevent new infection during pregnancy. However, finding one-sixth of women with parasitemia during pregnancy and the higher proportion (74%) of active placental malaria could suggest the limited efficiency of IPTp-SP. The limited efficacy of IPTp-SP could be explained by a higher prevalence of *P. falciparum* resistance to SP in the setting [130], which might have compromised the efficacy of SP to clear parasitemia, placental parasites and protect women against new infection [119].

4.2.2 Predictors of parasitemia during pregnancy, malaria and adverse birth outcomes at delivery

We further explored factors associated with parasitemia during pregnancy, malaria outcomes at delivery and anegative birth outcomes. Primigravida women were found to have almost two-fold significantly higher risk of parasitemia during pregnancy compared

to multigravida women (Table 3 of **Paper II**). In addition, adolescent pregnant women (<20 years) had 64% significantly higher chances of having placental malaria at delivery as compared to adult women aged 20-34 years both on univariate (p=0.014) and multivariate model p=0.016 (Table 3 of **Paper II**). On the contrary, having parasitemia during pregnancy did not significantly increase the odds of any adverse birth outcome (OR 1.27 [95% CI= 0.75, 2.15] p = 0.38) at delivery. Similarly, taking optimal IPTp-SP (≥3 doses) did not reduce the odds of placental malaria (Table 3 of paper II) at delivery compared to lower doses (<3) of IPTp-SP. On the other hand, optimal IPTp-SP (≥3 doses) significantly increased the mean birth weight by 195g (95% CI= 110 to 279) p < 0.001 and reduced the odds of low birth weight by 66% as compared to sub-optimal IPTp-SP (<3 doses) p=0.007.

We could not find a significant association of parasitemia during pregnancy with adverse birth outcomes in this sub-study. Contrarily, previous studies reported a significant association between parasitemia during pregnancy with adverse birth outcomes at delivery [191,192]. Evidence indicate that patent parasitemia during pregnancy is associated with negative birth outcomes despite treatment with highly effective antimalarial drugs [193]. Possibly, excluding participants with patent parasitemia (detected by mRDT) at enrollment might be one of the reasons for the lack of significant association between parasitemia during pregnancy with adverse birth outcomes in the present sub-study. In addition, the majority of parasitemia cases during ANC visit were sub-patent (misses by RDT but detected by PCR). The effect of sub-patent malaria during pregnancy on adverse birth outcomes is controversial. A study from Benin reported a significant association between sub-patent malaria and adverse birth outcomes [192], contrary to a study from Malawi, which did not find a significant impact of sub-patent malaria on adverse birth outcomes [191].

Receiving optimal (≥3 doses) IPTp-SP did not reduce the odds of placental malaria significantly compared to less than three doses of IPTp-SP. This data is comparable to other studies from endemic areas with high *P. falciparum* resistance to SP [104-106].

Nevertheless, we found improved birth weight significantly associated with optimal doses of IPTp-SP (≥3) than lower doses of IPTp-SP (<3) consistently with previous studies [99,194]. The impact of IPTp-SP on birth weight is thought to be contributed by factors more than just the antimalarial effect of SP. For instance, the antibacterial effect of SP [195] is hypothesized to partly contribute to the effect of IPTp-SP on birthweight [196]. Although we did not control for bacterial infections, the antibacterial effect of SP might have partly contributed to the observed improved birth weight considering the co-existence of malaria and bacterial infections during pregnancy in sub-Saharan Africa [197]. Nevertheless, this thesis reaffirms the significant impact of optimal IPTp-SP (≥ 3 doses) in improving birth weight from a setting with moderate malaria transmission intensity and high *P. falciparum* resistance to SP.

4.3 COMPARISON OF IPTp-DHP EFFECTIVENESS VERSUS THE STANDARD IPTp-SP FOR PREVENTION OF MALARIA IN PREGNANCY AND ADVERSE BIRTH OUTCOMES (PAPER III)

In Paper III we compared the effectiveness of monthly IPTp-DHP versus the standard IPTp-SP for the prevention of malaria and negative birth outcomes. We hypothesized that IPTp-DHP could be superior to IPTp-SP for the prevention of malaria in pregnancy and negative birth outcomes in a area with moderate malaria transmission intensity. We enrolled a total of 956 pregnant women who were 1:1 randomized to receive either IPTp-DHP (n=478) or the standard IPTp-SP (n=478) at each month. The distribution of primigravida and multigravida at baseline was not significantly different between IPTp-SP and IPTp-DHP groups (Table 1 of **Paper III**). Similarly, the mean age with one standard deviation at enrollment was not significantly different between IPTp-SP (26.6±7 years) and IPTp-DHP (26.8±8 years). Participants in both groups received a median of 3 (minimum=1, maximum=5) IPTp doses.

4.3.1 Malaria outcomes between the treatment groups

We found significantly higher protection for both symptomatic malaria and parasitemia during pregnancy in IPTp-DHP group compared to IPTp-SP group. Significantly, lower incidence of symptomatic malaria per person-year at risk was found in IPTp-DHP (0.02) compared to IPTp-SP (0.12) with PE=86% (95% CI=37 to 97) p=0.002. Similarly, significantly lower incidence of parasitemia per person-year at risk during ANC was observed in IPTp-DHP (0.28) as compared to the standard IPTp-SP (0.67) with PE=59% (95% CI=38 to 72) p<0.001. Further analysis using Kaplan Meir plot with log Rank test revealed a significantly lower risk of parasitemia overtime during ANC in IPTp-DHP than IPTp-SP p<0.001 (Figure 2 of **Paper III**).

Similarly, we found significantly lower prevalence of malaria outcomes at delivery in IPTp-DHP arm compared to IPTp-SP arm. The prevalence of our primary outcome (histopathological placental malaria) both active and past infection was significantly lower in IPTp-DHP (2.5%, 12/478) as compared to IPTp-SP (8.2%, 39/478) with PE=69% (95% CI=42 to 849) p<0.001. In our ad-hoc analysis stratified by active or past placental infection, IPTp-DHP was significantly associated with lower prevalence of active placental malaria (1.3%, 6/478) and higher PE (80%, 95% CI= 51 to 92) compared to the standard IPTp-SP (6.1%, 29/478) p<0.001 but not past placental malaria infection (Table 2 of **Paper III**). The presence of any malaria at delivery was significantly lower in IPTp-DHP (8.2%, 39/478) than in IPTp-SP (18.2%, 87/478) with PE=55 (95% CI=36 to 71) p<0.001. Similarly, we found significantly lower prevalence of parasitemia in placental blood, cord blood and maternal peripheral blood by RDT, microscopy and PCR in IPTp-DHP arm as compared to IPTp-SP arm (Table 2 of **Paper III**).

This thesis reports the superiority of monthly IPTp-DHP to IPTp-SP for the prevention of malaria in pregnancy from an area of moderate of malaria transmission intensity for the first time. We confirm that IPTp-DHP initiated either on the second or third trimester is

superior to IPTp-SP for the prevention of malaria in pregnancy. Our finding is comparable to previous trials which reported the superiority of IPTp-DHP to IPTp-SP for prevention of malaria in pregnancy when initiated early during the second trimester (≤ 20 weeks) [114,144] or second and third trimesters [113] but in areas with high malaria transmission intensity. Interpretation of these findings together, suggest that monthly IPTp-DHP may be suitable regimen to replace IPTp-SP in areas with high prevalence of *P. falciparum* resistance to SP.

4.3.2 Adverse birth outcomes between the treatment groups

We were interested in examining whether the superior effects of IPTp-DHP to IPTp-SP on malaria outcomes could be translated to a superior impact on anemia during pregnancy and negative birth outcomes. Using ITT analysis, we report a significantly lower prevalence of LBW (p=0.003) and higher mean birth weight (mean difference=55, 95% CI= 19 to 93g; p=0.004) associated with IPTp-DHP compared to IPTp-SP. However, we did not find a significant difference in anemia during pregnancy and composite adverse birth outcome (stillbirth, premature birth, spontaneous abortion, LBW and fetal anemia) between IPTp-DHP and IPTp-SP groups (Table 3 of **Paper III**).

The association between malaria in pregnancy with LBW is well established [198]. Thus, it may not be surprising to observe the superior effect of IPTp-DHP on malaria in pregnancy being translated to a superior effect on birth weight as compared to IPTp-SP. However, this finding contradicts previous trials [113,114,144]. Possibly, the differences in the design and the intensity of malaria transmission between the different settings could partly account for the observed difference of IPTp-DHP on LBW as compared to the standard IPTp-SP. Essentially, this thesis adds an evidence to the growing literature, for the first time showing the superiority of IPTp-DHP against LBW as compared to IPTp-SP from a moderate malaria transmission setting.

Furthermore, the lack of IPTp-DHP significant impact on anemia as compared to IPTp-SP in this thesis contradicts previous trials [113,114,144]. One reason to account for the observed difference might be the exclusion of women with patent malaria (detected by mRDT) at enrollment in our design. The association of patent malaria with anemia is well known. Therefore, excluding women with patent malaria in our design might have limited the impact of IPTp-DHP on malaria-associated anemia. Also, differences in other causes of anemia in different geographical settings might have partly contributed to the observed difference.

4.3.3 Implication of the findings to the Sustainable Development Goal number 3

The need for the superior drug for IPTp is inevitable considering the adverse effects of pregnancy-associated malaria in utero and beyond uterine life. Recent evidence indicates that placental malaria is associated with a significantly higher risk of malaria [199] and non-malaria infections during infancy and childhood [200]. The possible mechanism for this association could be the fetal immune tolerance caused by regulatory T cells when

exposed to malaria antigens in utero persisting to infancy and childhood [76]. Both malaria and non-malaria infections are the most common causes of infant and child mortality in sub-Saharan Africa [201-203]. Therefore, our findings suggest that monthly IPTp-DHP will not only reduce malaria in pregnancy and associated LBW but also could contribute to the achievement of global SDG number 3.2, targeting to end deaths that could be prevented among newborns and children below 5 years of age by 2030 [186].

4.4 PHARMACOGENETICS AND PHARMACOKINETICS OF PIPERAQUINE AND THEIR RELEVANCE ON TREATMENT OUTCOMES DURING IPTp WITH DHP (PAPER IV)

In **Paper IV** we investigated the pharmacogenetics, day-7 pharmacokinetics of piperaquine and their association with treatment outcomes during IPTp with DHP. Our hypothesis was based on the fact that IPTp-DHP did not completely eliminate parasitemia during pregnancy (**Paper III**). We then postulated that the observed parasitemia in IPTp-DHP group would possibly be contributed by inter-individual variations in plasma piperaquine concentration associated with both genetic and non-genetic variations. We prospectively followed pregnant women (n=446) who received monthly IPTp-DHP in a two arm randomized clinical trial (**sub-study III**). From these women, we collected samples for piperaquine pharmacogenetics and followed them for day-7 pharmacokinetics after each monthly IPTp-DHP dose, malaria and negative birth outcomes till delivery. The baseline characteristics are summarized in Table 1 of **Paper IV**.

4.4.1 Day-7 piperaquine pharmacokinetics, pharmacogenetics and their associations with IPTp-DHP outcomes

We observed that the repeated administration of IPTp-DHP at each month caused a significant increase in plasma day-7 piperaquine concentration over time. Using paired t-test, we found significantly lower geometric mean day-7 piperaquine concentration after receiving the first IPTp-DHP compared to geometric mean day-7 piperaquine plasma concentration after receiving the second IPTp-DHP dose ($p<0.001$). Similarly, the day 7 piperaquine geometric mean difference between after receiving the second and the third IPTp-DHP doses was significantly different being higher after receiving the third IPTp-DHP dose (Figure 1 of Paper IV). We also noticed that few women had day-7 piperaquine concentration below the threshold (30ng/mL) established for treatment success after the first (6.1%, 15/245), second (4.1%, 5/122) and third (3.6%, 2/55) IPTp-DHP doses.

We presented our results for the observed genotypes and allele frequency in Table 3 of **Paper IV**. We did not detect *CYP2C8*3* allele in our study population from Tanzania. In this population, all observed allele frequencies were in agreement with Hardy-Weinberg equilibrium (Table 3 of **Paper IV**).

The observed significant increase in mean day-7 concentration of piperaquine associated with repeated IPTp-DHP given monthly is comparable to previous IPTp studies which reported a significant increase in piperaquine trough plasma concentration over time during monthly IPTp with DHP [158,159]. As a limitation to this sub-study, overall piperaquine exposure was not assessed. However, a recent study reported that the predicted peak plasma concentration of piperaquine was not altered significantly despite the significant increase in plasma trough concentration with repeated IPTp-DHP [159]. This may suggest that the significant accumulation of plasma piperaquine with repeated IPTp-DHP could be safe but providing optimum protection against malaria and associated LBW. In addition, the high percent of women who attained optimum day-7 piperaquine

concentration (≥30ng/mL) further support that IPTp-DHP given monthly could provide optimal plasma piperaquine concentration needed for sufficient malaria protection in more than ninty percent of women.

We then explored the impact of pharmacogenetic variations, piperaquine day-7 pharmacokinetics and baseline characteristics on parasitemia during pregnancy and malaria and adverse birth outcomes at delivery. Using Cox regression model, we observed significantly higher risk of parasitemia at ANC overtime during pregnancy (HR= 5.22 95% CI=1.70 to 16) in pregnant women with lower (<30ng/mL) day-7 piperaquine concentration when compared to women who attained concentration ≥30ng/mL after receiving the first IPTp-DH dose (p=0.004). We also observed a similar non-significant trend after the second IPTp-DHP dose (HR= 4.40 95% CI=0.94 to 20) p=0.05. We examined this association graphically on Kaplan Meir plot with Log Rank test. In this analysis, the risk of parasitemia during ANC overtime was observed to be significantly higher in women with lower day 7 piperaquine concentration (<30ng/mL) compared to those with higher concentration (≥ 30ng/mL) both after the first (Log Rank p=0.002) and the second (Log Rank p=0.02) IPTp-DHP doses (Figure 4 of **paper IV**).

On the contrary, we did not find significant association between genetic variation in *CYP3A4*1B, CYP3A5*, and *CYP2C8*, and baseline characteristics with risk of parasitemia during pregnancy (Table 6 of **Paper IV**). Equally, baseline characteristics and *CYP3A4*1B, CYP3A5* and *CYP2C8* genotypes were not significantly associated with any parasitemia and adverse birth outcomes at delivery.

This thesis report for the first time the association between piperaquine pharmacogenetics and day-7 pharmacokinetics with treatment outcomes during IPTp with DHP. The observed significantly higher risk of parasitemia associated with lower day 7 piperaquine concentration is comparable to previous studies which reported the association of lower day-7 piperaquine concentration with treatment failure in treatment regimen [204-206]. Our result implies that the pre-established target day-7 piperaquine concentration (30ng/ml) [159] for the treatment regimen could also be used for DHP surveillance in IPTp regimen.

4.4.2 Predictors of day-7 piperaquine pharmacokinetics

We examined the impact of pharmacogenetics variations and baseline characteristic on day-7 piperaquine pharmacokinetics. We found significantly lower geometric mean day 7 piperaquine concentration in women with defective *CYP2C8* allele compared to women with *CYP2C8*1/*1* genotype after the second IPTp-DHP dose (Figure 2 and Table 4 of **Paper IV**). Also, we observed a similar non-significant trend after the first IPTp-DHP dose p=0.27 (Figure 2 of **Paper IV**). Using linear mixed model, we found 5% non-significant lower change in log day 7 piperaquine concentration in women with at least one defective *CYP2C8 (*2 or *4)* allele compared to wild type p= 0.11 (Table 5 of **paper IV**). We did not observe a significant association between day-7 piperaquine concentration with genetic variations in *CYP3A4*1B* and *CYP3A5* (Table 4 of **paper IV**).

Using linear mixed model, we observed a significant association between primigravida and lower change in monthly day-7 piperaquine concentration as compared to multigravida p=0.04 (Table 5 of **Paper IV**). Bodyweight and trimester at enrollment were not associated with a significant effect on change in day-7 piperaquine concentration (Table 5 of **Paper IV**).

Data in the literatures regarding the pharmacogenetics-pharmacokinetics relationship for *CYP2C8*2* which is predominantly found in black population are largely unavailable contrary to data regarding *CYP2C8*3* found in white population [207-213]. Current evidence concerning *in vivo* effects of *CYP2C8*3* genotype are disagreeing with each other. Some studies reported significantly lower plasma concentration indicating increased metabolism of drugs [210,213-215] while others reported higher plasma concentration suggesting reduced metabolism [208,211,212] associated with defective *CYP2C8*3* alleles as compared to the wild type. It is hypothesized that the activity of *CYP2C8* alleles could be substrate-specific.

In this thesis, significant association between day-7 piperaquine plasma concentration with *CYP2C8* genotypes was found from a population with dominant *CYP2C8*2* allele. We observed lower day-7 piperaquine concentration in women with at least one defective *CYP2C8* allele after the first and second monthly doses of IPTp-DHP than in homozygotes wild type. The association was significant after the second IPTp-DHP dose with lower concentration in women with *CYP2C8*2/*2* genotype compared to those the wild type genotype. Notably, our exploration on the trend of plasma piperaquine concentration change with repeated monthly IPTp-DHP doses revealed significantly higher increase in participants with *CYP2C8*2/*2* genotype than in participants with one defective *CYP2C8 (*2 or*4)* allele and *CYP2C8*1/*1* genotype (Figure 3 of **Paper IV**). The accumulation of plasma piperaquine concentration with repeated monthly IPTp-DHP and pregnancy-associated alterations in expression of CYP enzymes could modify the metabolic activity in *CYP2C8* genotype. This thesis identified the potential impact of *CYP2C8* genotypes on the pharmacokinetics of piperaquine during IPTp which warrant further studies for more evidence.

This thesis did not find significant impact associated with *CYP3A4*1B* and *CYP3A5* genetic variations on day-7 piperaquine pharmacokinetics. Our result is comparable to one study which reported a similar non-significant association between *CYP3A4*1B* and *CYP3A5*3* genotypes with piperaquine pharmacokinetics from Cambodia [162]. This finding suggests that monitoring of *CYP3A4*1B* and *CYP3A5* pharmacogenetics may not be important during IPTp with DHP.

5 CONCLUSIONS AND PERSPECTIVES

5.1 CONCLUSIONS

This thesis evaluated the effectiveness of IPTp for the prevention of malaria and negative birth outcomes in a setting with high parasite resistance to SP and moderate malaria transmission intensity. Here, I describe my contribution to the growing evidence indicating the significant impact of IPTp-DHP on malaria in pregnancy and LBW as compared to the standard IPTp-SP.

Firstly, we reported that asymptomatic parasitemia and associated anemia is common at the first ANC before initiating IPTp. We also observed higher burden of asymptomatic malaria and anemia in primigravida and adolescent pregnant women, similar to other findings in sub-Sahara Africa.

Moreover, we observed substantial rates of parasitemia, placental malaria and associated adverse birth outcomes in pregnant women receiving the standard monthly IPTp-SP. We found that receiving optimal IPTp-SP did not prevent placental malaria but improved birth weight compared to lower IPTp-SP doses.

In addition, we reported the superiority of monthly IPTp-DHP to IPTp-SP for the prevention of malaria in pregnancy in areas with moderate malaria transmission intensity, similar to the findings from areas with high malaria transmission intensity. Also, we found for the first time, the superiority of IPTp-DHP on malaria translated to superior effects on LBW as compared to IPTp-SP. The current data taken together with the previous findings support the hypothesis that monthly IPTp-DHP could replace IPTp-SP in areas with high *P. falciparum* resistance to SP.

Finally, we reported for the first time, the association of lower day-7 piperaquine concentration with the risk of parasitemia during pregnancy. We also identified the potential impact of *CYP2C8* genotypes on piperaquine pharmacokinetics.

5.2 FUTURE PERSPECTIVES

Although my PhD thesis has responded to several research questions, some other areas in this field require further investigation.

- For instance, we observed more than half of asymptomatic parasitemia as sub-patent infection (not detected by RDT but detected by PCR). Currently, the impact of sub-patent malaria on adverse birth outcomes are inconclusive. It would be nice to further investigate the effect of sub-patent parasitemia on adverse birth outcomes to give more evidence. This will inform policymakers and help to improve the control of malaria in pregnancy. A systematic review and meta analysis on the available few literatures could give a clue. Moreover, it would be interesting to quantify the role of asymptomatic malaria in pregnant women on the overall malaria

transmission, especially in areas targeting elimination. This would help to revise and improve elimination strategies.

- Additionally, we observed that optimal IPTp-SP did not prevent placental malaria but improved birth weight as compared to sub-optimal IPTp-SP. The antibacterial effect of SP has previously been hypothesized to partly contribute for the observed improved birth weight [195]. However, the exact mechanism is not yet known. It would then be interesting to scrutinize the exact mechanism and describe why lower doses (<3) of IPTp-SP would not be associated with improved birth weight.
- Furthermore, we found that IPTp-DHP is superior to IPTp-SP for the prevention of malaria and LBW. It would be important to further investigate the feasibility and adherence of IPTp-DHP. This will help policy decisions for IPTp especially in areas with high SP resistance.
- We also found that IPTp-DHP was not superior to IPTP-SP on composite adverse birth outcomes. However, in sub-Sahara Africa adverse birth outcomes could also be associated by infections other than malaria [216]. Considering the reported antibacterial effect of SP, it would be interesting to investigate whether combining monthly IPTp-DHP with SP would be superior to IPTp-DHP alone or IPTp-SP for prevention of composite adverse birth outcomes.
- In this thesis, we reported the superiority of IPTp-DHP to IPTp-SP among pregnant women on their second and third trimesters who are currently eligible for IPTp. However, pregnant women on their first trimester have similar risks of malaria and associated negative birth outcomes. Recent evidence indicates that artemisinin-based combination therapy is safe during the first trimester [217]. It would then be interesting to explore the safety and efficacy of IPTp-DHP during the first trimester.
- We have also found a significant association between lower day 7-piperaquine concentration (<30ng/mL) with a higher risk of parasitemia, indicating that this target concentration could be a predictor of IPTp-DHP effectiveness. However, this thesis reported data from an area with no reported emerging DHP resistance [218]. Thus, it will be interesting to investigate if this target concentration could also be applied to areas where reduced parasite sensitivity due to emerging DHP resistance was reported [219,220].
- In this thesis, we identified for the first time a significant association between *CYP2C8* genotypes with piperaquine pharmacokinetics. It would be interesting to further explore the impact of *CYP2C8* on piperaquine pharmacokinetics and clinical outcomes during IPTp with DHP in a relatively large sample size to provide more evidence.

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